

A belated agreement that requires faster progress

The treatment of contract and postdoctoral researchers by employers is notoriously casual. A year-old initiative to improve matters in Britain may provide an example to others, but has a long way to go.

Some professors have not even heard of it. Some are implementing it conscientiously. Those in universities who are supposed to benefit are beginning to — but by no means enough of them, and to too little an extent. Those employed by the government are seeing little progress.

"It" is a concordat, announced almost exactly a year ago today, between British university vice-chancellors and research funding councils. It is intended to underpin the provision of employment rights and support for "them" — the great underclass of research in Britain: postdoctoral and other contract researchers. Recent articles and correspondence in *Nature* suggest that Britain is far from being the only country in which these researchers are relied upon while simultaneously undervalued. Yet still young scientists apply in droves for postdoctoral research posts.

The concordat does not address pay scales, in respect of which conditions of postdoctoral employment have recently improved in UK universities. Rather, it is intended to ensure that contract researchers obtain just treatment in maternity leave, salaries that reflect experience and seniority, adequate training and conscientious monitoring of progress and career advice.

Soundings within the UK university community suggest that progress with the concordat is patchy. The scheme began to be implemented only six months ago. All publicly funded universities and research councils must implement it and at senior levels are indeed doing so. But what goes on at the top of an institution does not always readily translate into action three or four levels down. Issues are already emerging about which more action is necessary.

One concerns a tendency for academics, seeking to employ

researchers on a contract, to apply for grants and bid low down the pay scale, even if the researcher merits more. There are signs that this is happening despite an explicit allowance within the concordat for research councils to provide enhanced pay where appropriate. Unless grant applicants are given stronger encouragement in this regard, experienced senior contract staff will continue often to be underpaid.

The provisions for supporting maternity leave appear to be working. But a researcher who moves from one contract to another, even if funded by the same research council but at a different university, comes under a new employer. He or she loses employment rights that would have been accrued had he or she stayed still.

All that being said, postdoctoral researchers in universities are experiencing significantly more progress than those employed directly by the very body that backed the concordat in the first place — the British government. Research councils merit scrutiny here. The Medical Research Council, with an increasing proportion of contract researchers on its books as older permanent researchers retire, announced last October its intention to pursue concordat-like measures for its staff. It admits that it still has some way to go. The Natural Environment Research Council's enlightened policy is that any employee of more than five years' standing should be permanent. Unfortunately, many such employees are still impermanent.

The concordat is a step towards righting a scandalous state of affairs that persisted for far too long. Its example should stimulate researchers in some other countries to demand similar measures. Every means should be found to keep up the pressure on all employers who keep contract researchers, doing work of central importance, in a state of unjust personal disadvantage. □

Discovery delocalized

The timing of celebrations of J. J. Thomson's discovery of the electron is necessarily arbitrary.

The two-slit experiment shows how the same electron pops up, disconcertingly, in at least two places at once. Such positional uncertainty applies to the discovery of the electron itself. On 21 and 22 March, the University of Cambridge celebrates the centenary of that achievement by its very own J. J. Thomson. Thomson announced the finding at the Royal Institution on 30 April 1897, but Cambridge's error in celebrating a centenary after only 99.98 years is as a proportion less than those of Thomson's original estimates of the mass and charge of his 'corpuscle'. It is in fact doubly pardonable, as the centenary adds a historical resonance to this, Britain's National Science, Engineering and Technology week.

Although posterity has awarded the crown for the discovery to Thomson, Emil Wiechert gave the first account of a negatively charged atomic constituent to the Königsberg Physikalisch-ökonomische Gesellschaft on 7 January 1897 — Thomson did not publish his findings until October. Either of those dates could serve as the centenary. Being the reputable journal that we are, we could suggest that celebra-

tions be postponed until the publication date of October, rather than being pinned to a Victorian equivalent of a press conference in April.

In truth, the timing does not matter. No discovery is made in isolation, and indeed the finding of the electron was a less singular event than many other discoveries of fundamental entities. Arguably, it began with the first quasi-description of the thermionic effect, by du Fay in 1733, and included Millikan's work on the charge on the electron in the 1930s. Furthermore, as Steven Weinberg describes on pages 213–215, the discovery of the electron is still unfolding: although its mass and charge are well established, nobody yet knows why these properties have the values they do — although string theorists are working on it. In short, from a truly Olympian viewpoint, the uncovering of the electron might be seen as akin to the classic quantum description of a single free elementary particle — an unbounded field of endeavour, for which one date of commemoration is as good as any other. But, some time in 1897, we became confident that, whatever it truly is, it is there. □



Panel seeks 'fundamental' shift in handling of observation data

[WASHINGTON] The US Mission to Planet Earth (MTPE), which includes a multibillion-dollar series of satellites to observe the planet, oceans and atmosphere, is undergoing yet another major review that may complete the programme's evolution from the use of large, centralized spacecraft to a smaller and more distributed system.

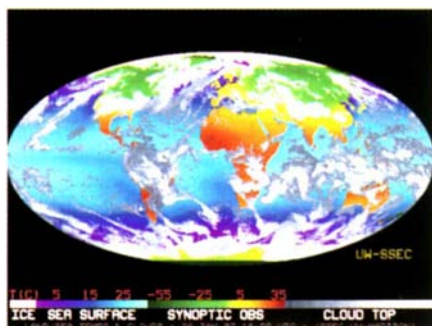
One early result of the review is "an absolutely fundamental change in philosophy" as to how data from the Earth Observing System (EOS) of satellites are disseminated, according to Steven Wofsy, an environmental scientist at Harvard University and the new chair of NASA's advisory group for Earth science.

Since it began in 1991, the MTPE has been revised on many occasions, its spacecraft have shrunk in size, and its projected budget has dropped from \$18 billion to just over \$6 billion. But the current issue is not money. NASA is revamping the programme at a time when the budget appears stable and the first launch — of the AM-1 spacecraft — is only a year away.

Michael Mann, the agency's deputy administrator for Earth science, says the purpose of this first 'biennial review' is to develop a strategy for the mission "that allows us to be flexible" and to respond to criticisms and recommendations from outside groups.

The latest — and probably bluntest — such criticism came recently from Wofsy's panel, which is part of the NASA Advisory Council. In a letter sent last week to Daniel Goldin, NASA administrator, the council made three pointed recommendations.

First, it said there should be a "fundamental change" to the EOS Data and Information System (EOSDIS) designed to



Global vision: satellite data such as that in this temperature map have become invaluable

process, archive and distribute data from dozens of orbiting instruments. "The requirements should be limited and realistic, the architecture should be open, and the data products should be largely the responsibility of the principal investigators."

The EOSDIS is behind schedule and has suffered technical problems, most recently when it failed a major integration test early this year. The data system, which before the current review was projected to cost as much as \$2 billion, has been singled out for reform by other review panels. They have advised NASA to move away from large, centralized databanks to a "federation" of smaller data providers (see *Nature* 377, 191; 1995).

NASA is committed to having some kind of EOSDIS in place for the AM-1 and Landsat 7 missions, says Mann. But it has suspended work on subsequent versions. The EOSDIS could even be scrapped altogether, with scientists simply releasing processed data from individual instruments over the Internet.

Wofsy's panel argues that whatever system takes its place should serve scientists

first. "Those components [of the current system] that do not meet limited requirements for defined scientific and applications users should be cancelled or modified." Critics say one of the problems with the current EOSDIS is that it has been asked to serve too many masters, from scientists to schoolchildren to the general public. Mann says non-scientist "customers" might still be provided for, depending partly on the cost.

The advisory council also recommends — and NASA has tacitly agreed — that all EOS missions after PM-1 in 2000 (for which hardware is already being built) should be "completely re-examined" to favour smaller, faster missions that can take advantage of new technology and new scientific thinking.

The programme may even set a new competition to provide the instruments to be flown on CHEM-1, the next spacecraft in the series, even though some investigators have been working on the project for nearly a decade. Wofsy says the MTPE will benefit from continually re-evaluating the scientific questions it asks, and from being able to fly a greater number of "hypothesis-driven missions" with shorter turn-around times. One model for this approach is the new low-budget Earth System Science Pathfinder series of missions, the first two of which were to be selected this week.

Finally, says the panel, next year the MTPE programme should shift from its heavy emphasis on spacecraft observations to "substantially increase the resources devoted to in-situ and process studies, modelling and analysis". In other words, less money should be spent on orbiting hardware, and more on ground- and aircraft-based research.

Yet to be decided is how all these reforms will affect one of the EOS system's original goals, namely to gather the same key measurements from the same set of instruments flying in space over many years, in part to avoid calibration problems that make it difficult to work out long-term trends.

Some supporters of the MTPE worry that such a sweeping re-evaluation of the programme as it approaches its first launch signals confusion and a lack of clear goals. Others applaud the new flexibility as a sign of progress, a liberation from NASA's old practice of flocking in technologies and scientific strategies years ahead of a launch, which bars new ideas.

That debate is certain to be taken up in Congress this year. Hearings on the Mission to Planet Earth were due to be held before the House Science Committee's subcommittee on space this week.

Tony Reichhardt

Senate bill seeks controls on genetic data

[WASHINGTON] A far-reaching bill that would bar employers and insurers from discriminating on the basis of genetic information was introduced into the US Senate last week by Pete Domenici (Republican, New Mexico).

The bill has been welcomed by consumer and privacy advocates. But it is opposed by many researchers, who argue that it would impose new responsibilities and procedures on them. Francis Collins, director of the National Human Genome Research Institute, called the bill impracticable.

The Genetic Confidentiality and Nondiscrimination Act of 1997 would also require any third party to obtain a donor's informed and written consent to "collect,

store, analyze or disclose an individual's genetic information". The bill's wording has been slightly revised from a 1996 version; for example, a provision establishing an individual's ownership of his or her DNA has been deleted.

But some researchers say that the bill would still cripple research by drastically reducing access to tissue samples from the country's vast library of stored pathological specimens. The bill is "unacceptably restrictive and burdensome," says one.

The bill has been referred to the Senate Labor and Human Resources Committee, whose chairman, James Jeffords (Republican, Vermont), is one of the bill's co-sponsors.

Meredith Wadman

US senators urge caution on cloning ban

[WASHINGTON] Several influential US congressmen last week urged their colleagues to take a calmer approach to human cloning, after an initial burst of support for restrictive legislation that followed last month's announcement that a sheep had been cloned.

But at the end of the week, the 18-member National Bioethics Advisory Commission (NBAC), which has been asked by President Bill Clinton to draw up policy recommendations on human cloning, heard a wide range of views, from calls to treat human cloning as simply another form of assisted reproductive technology to "grave concerns" from animal rights activists about cloning's implications for animal welfare.

Separately, in California, State Senator Patrick Johnston introduced a bill banning human cloning research and experiments for five years. But the office of Republican governor Pete Wilson's cautioned against a "knee-jerk action" that could threaten valuable research.

Among the national politicians urging action about legislation was John Porter (Republican, Illinois), chair of the subcommittee in the House of Representatives that funds the National Institutes of Health (NIH). Porter said that "cloning itself can lead to a great deal of progress in science", and added: "I think we should simply take a good, hard, long look at it."

Such sentiments were echoed by Connie Mack (Republican, Florida), a key Senate supporter of biomedical research, and Senator Bill Frist (Republican, Tennessee), the heart surgeon who heads the public health and safety subcommittee of the Senate Labor

and Human Resources Committee.

Frist compared the current uproar to the public reaction to the first heart transplants. He said that he opposed any legislation that would block the potential benefits of human cloning research for diseases such as cystic fibrosis and sickle-cell anaemia.

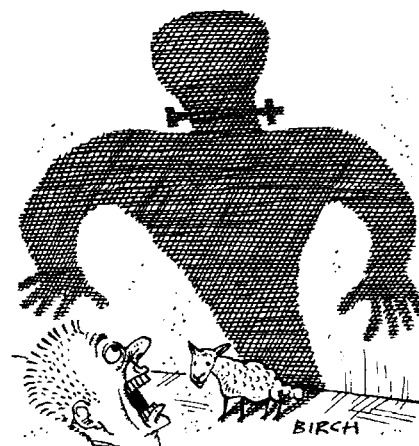
Since the news of the sheep cloning was reported, Clinton has banned federal funding for "cloning of human beings". Two bills have been introduced in Congress that would ban federal funding for human cloning, and another would impose a \$5,000 civil penalty on anyone doing such work (see *Nature* 386, 97; 1997).

Appearing before Frist's subcommittee last week, Ian Wilmut, who led the team of scientists who cloned the sheep at the Roslin Institute in Edinburgh, endorsed proposals for an international ban on human cloning, and said he took comfort in existing British legislation on this issue.

While urging senators not to "throw out this baby with the bath water" by enacting legislation that would impose excessive restrictions on research, he pointed out that "many of the most important experiments could more readily be carried out" with laboratory or farm animals.

As for cloning humans for reproductive purposes, Wilmut said he had never heard of a reason for this that he found ethically acceptable. When Tom Harkin (Democrat, Iowa) called such caution "utter nonsense" and predicted human cloning in his lifetime, Wilmut said: "I hope you're wrong."

At the same hearing, Harold Varmus, the NIH director, urged the senators not to legislate. He credited the existence of a "vibrant"



US biotechnology industry directly to the lack of legislation on recombinant DNA technology when it first emerged. "When I hear about research on human cloning as something that ought to be taken away, I shiver," said Varmus.

But Christopher Bond (Republican, Missouri), who last month introduced a bill banning federal funding for research related to the cloning of humans, told the hearing that this topic did not need to be studied. "There are aspects of human life that should be off limits to science," he said.

Separately, the NBAC heard two days of testimony from religious figures, ethicists, scientists and the public. Leon Kass, a professor of social thought at the University of Chicago, told the commission that human cloning is "radically new" and dangerous.

Kass said that human cloning would be impossible without unethical experiments, and presents an unacceptable threat to human identity and individuality. As a technique for turning "procreation into manufacture", he argued that cloning represents an attempted human control of progeny that is "inherently despotic".

But two ethicists disagreed. John Robertson, a professor at the University of Texas at Austin, argued that a ban on cloning would unacceptably infringe scientific and reproductive freedoms which he said are protected by the US constitution.

He said that cloning was "much less ominous" than the manipulation of human genomes, as it simply duplicates an existing genome. In the absence of demonstrable harm to others, cloning should receive the same protection as other non-coital methods of assisted reproduction, he argued.

Ruth Macklin, a bioethicist at Albert Einstein College of Medicine in New York, said that it will be crucial to distinguish between cellular level cloning for research purposes and cloning with the aim of producing a human being. She urged the government to develop a regulatory framework to control human cloning research. **Meredith Wadman**

WHO chief defends use of animal models

[GENEVA] Hiroshi Nakajima, director-general of the World Health Organization (WHO), warned last week that opposition to human cloning should not lead to an indiscriminate ban on all cloning procedures and research, and praised the potential benefits of animal cloning to human health.

In a statement released by WHO headquarters in Geneva, Switzerland, Nakajima condemned the use of cloning to replicate humans as "ethically unacceptable", as it would violate some of the basic principles governing medically assisted

procreation.

But he pointed out that the cloning of human cell lines is a routine procedure in the production of monoclonal antibodies for diagnosis and research on diseases such as cancer. "Animal cloning also offers opportunities to advance research on diagnosis and treatment of diseases affecting human beings," Nakajima said.

The implications of the recent experiments at the Roslin Institute in Scotland will be discussed next month at a meeting of the scientific and ethical review group of the WHO's special

programme on research, development and research training in human reproduction.

Meanwhile, Edith Cresson, the European Commissioner responsible for research, told the European Parliament last week that there should be a total ban on cloning human beings – a proposal that was unanimously endorsed by the parliament. But Cresson said that there should be common agreement in Europe on human cloning, where opinions already differed widely, before attempts were made to establish a global agreement. □

EU research ministers lean on Brussels

[LONDON & MUNICH] The European Commission is coming under pressure from member states of the European Union (EU) to impose tighter constraints on research projects to be funded under the fifth Framework programme (FP5), and to reduce its proposed emphasis on untargeted generic and basic research.

Their demands are contained in letters which are being sent to Brussels containing the comments of the various governments on the commission's latest draft proposals on FP5, which is due to last from 1999 to 2003, with a budget of more than ECU15 billion (US\$13.2 billion).

Unsurprisingly, some of the strongest criticism has come from the United Kingdom. Ian Taylor, Britain's minister for science, has written to Edith Cresson, the commissioner responsible for research, arguing against supporting any generic research that is not linked to targeted 'key actions'.

But Britain is not alone. Several other countries have expressed similar concern that, as the FP5 proposals stand, the goals of its research programmes are not sufficiently focused. The programmes reflect Cresson's desire to see that EU research projects meet a set of broadly defined social goals.

Germany is pressing for the 'key actions' proposed by the commission to have much sharper goals. Like Britain, it wants the com-

mission to set up advisory panels that would help to produce a precise description of such actions and to oversee their implementation.

Much of the criticism from EU states focuses on the commission's proposal that there should only be three main 'thematic programmes', aimed respectively at the sciences of the living world and the ecosystem, the 'user-friendly information society', and competitive and sustainable growth (see *Nature* 386, 5; 1997).

Several countries argue that the number of thematic programmes should be increased to between five and seven. Some argue that there should be thematic programmes devoted to the problems of environmental pollution and the life sciences. Another proposal is for a programme devoted to energy needs. Germany suggests that this should include two specific 'key actions', one aimed at the development of efficient, high-performance power plants with low carbon dioxide emissions, and the other on solar heating of buildings.

A second area of contention is the commission's proposal that it should set aside part of the funds for FP5 to support "general activities for the development of generic technologies and basic research". The commission argues that this is needed to develop the EU's general scientific and technological capability, but several member states say that this goal

can be achieved more effectively by channelling funds through national agencies.

Taylor of the United Kingdom, for example, expresses disappointment that the proposed focus on 'key actions' is being "potentially undermined" by the "continuing parallel emphasis" in the commission proposal on broadly based, non-targeted general activities for the development of generic technologies and basic research.

Germany wants basic research to be removed from the funds allocated to generic technology, and for such research to be funded only as a component of key actions.

France is believed to share many of Britain and Germany's concerns about the apparent breadth of the proposals. French officials are also said to be less insistent than they were previously that FP5 should allocate a substantial fraction of its funds to basic research, apparently accepting that it is more appropriate for such funds to be distributed nationally.

Drawing on the comments it is receiving, the commission is planning to produce a final draft of its FP5 proposals before mid-April. This will be submitted for approval both to the Council of Ministers — representing member states — and to the European Parliament. Agreement must be reached between all three bodies before the programme can proceed.

David Dickson & Alison Abbott

Political constraints restrict moves to conserve fish stocks

[LONDON] European fisheries and environment ministers ended a two-day meeting in Bergen, Norway, last week on protecting North Sea fish stocks by agreeing that urgent steps are needed to stop overfishing — but they failed to agree on a timetable for action.

The final declaration was a compromise between environment ministers, who favour prompt action, and the fisheries directorate of the European Commission, whose officials had pointed out that the European Union's Common Fisheries Policy remains the only legal mechanism for changing fishing practices.

Environmental groups and other observers believe that this restriction, if applied rigorously, renders the ministerial statement little more than "a pious form of words", as the fisheries policy is not due for review for five years.

The lack of specific targets and deadlines to combat overfishing, as well as the fact that the ministers' statement carries no legal force, prompted a chorus of derisory comments from environmental groups. But there was praise for Denmark, whose environment minister, Svend Auken,

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Caught in the act: Greenpeace protests.

announced that his country would suspend industrial fishing in "sensitive" areas.

A joint statement by Greenpeace, the World Wide Fund for Nature, BirdLife International and Seas at Risk says the declaration remains fundamentally flawed. "None of the actions can be achieved without precise targets and the reintroduction of specific deadlines."

Lord Perry of Walton, a member of the UK House of Lords Select Committee on

Science and Technology, says the only hope now is for governments to lobby for an earlier review of the Common Fisheries Policy. "The United Kingdom takes over the presidency of the EU shortly. One of the first things we should do is to push for the review to be advanced to 1998 or 1999."

The Lords committee reopened hearings on the fisheries issue two weeks ago, one year after its report on fish stocks management called for an immediate 30 per cent reduction in fishing.

Lord Selborne, the committee's chairman, said in a recent letter to the UK fisheries minister that, despite the report's warning of the dangers of overfishing, present EU fisheries policies displayed "a lack of urgency".

Unsustainable fishing is now a serious problem in the North Sea. Increasingly powerful fleets are catching younger and younger fish, often before they have time to reproduce, which has led to a crisis in the cod population (see *Nature* 385, 521–522; 1997).

Each EU country is assigned a 'catch quota' under the joint fisheries policy. But quotas are difficult to adhere to, as nets tend to catch a variety of species. Ehsan Masood

Harvard faces questions on missing data

[SAN DIEGO] The US Department of Veterans' Affairs is conducting an inquiry into whether the absence of documentation on key experiments leading to the publication of research carried out at a centre affiliated to Harvard Medical School should be treated as scientific misconduct.

Although internal investigations at Harvard last year led university officials to the conclusion that "it was not possible to ascertain whether the experiments had been done as described in the paper," they also decided that absence of documentation did not constitute scientific misconduct.

Officials of the veterans' affairs department, which funded the research, are now conducting their own investigation and reviewing the Harvard inquiry. Meanwhile, another federal agency is investigating a claim by a researcher that she lost her job after she questioned the paper when it was being submitted for publication.

The research concerns a genetic defect causing a Factor VII deficiency in an Italian patient. It was carried out under the direction of Kenneth A. Bauer, chief of haematology-oncology at the Veterans' Affairs Medical Center at West Roxbury, Massachusetts. The disputed experiments were carried out by Arnaldo A. Arbin, a physician from the

University of Milan who was doing research in Bauer's laboratory, and published last June in the journal *Blood*. Arbin has since moved to Yale University.

Bauer and Arbin say they received written reprimands from Harvard faculty authorities for the shortcomings in their data. Arbin admits that there was no laboratory notebook entry to document the questioned experiments, but says that this was because they were repetitions of earlier work. "In retrospect it obviously was a mistake," says Arbin. "But this was a completely innocent omission."

Bauer denies that there was any attempt to falsify data or any "wilful misrepresentation of data". He calls the missing data "an inexplicable lapse".

In a letter last October to the researcher who questioned the paper, Margaret L. Dale, an attorney in Harvard's Office of Research Issues, admitted that "there was concern that a paper had been published reporting on experiments for which there were not extant primary data". But Dale wrote that there was not sufficient evidence to conclude that there was scientific misconduct.

Dale pointed out that the experiments "were repeated in September under the supervision of an independent observer. As a

result of that process, it was determined that the paper could stand uncorrected." Peter V. Tishler, a Harvard physician who is associate chief of staff for education at the veterans' affairs department in West Roxbury, said he observed the repeating of the experiments.

The issue of whether the absence of primary data notebooks constitutes scientific misconduct has varied over the years. In a case in the late 1980s involving a former biochemist from Baylor College of Medicine in Houston, Texas, who made use of research results from a paper he was reviewing, the National Institutes of Health (NIH) decided that the absence of primary data was an important factor in finding misconduct.

But Christopher B. Pascal, acting director of the NIH's Office of Research Integrity (ORI), says that recent decisions show "the mere absence of records alone isn't sufficient proof of scientific misconduct". Pascal says that NIH is concerned that an individual might use a lack of records as an excuse for "dumping records" to cover up the fabrication of data. "The problem is, you have to prove intent," he says.

Paul M. Hoffman, the veterans' affairs department's director of medical research, says he is unable yet to make a judgement on whether the absence of primary data was scientific misconduct in the Harvard case. "It makes good sense that you should be able to document what you are publishing," says Hoffman, a neurovirology researcher. "If you can't, you can publish anything."

The same point is made by Dinah K. Bodkin, the former researcher in Bauer's laboratory who expressed concern about the Arbin paper. "Harvard has stood the definition of scientific misconduct on its head," she says.

Danielle Brian — executive director of the Project on Government Oversight in Washington, DC, which is assisting Bodkin — calls Harvard's conclusion about the lack of notebooks "patently absurd". In seeking a thorough investigation by the veterans' affairs department, Brian wrote that Harvard's handling of the case "appears to fly in the face of overwhelming facts". Don Gibbons, a spokesman for Harvard, says that the university thoroughly investigated the complaint before issuing its opinion last October.

Bodkin, who was not a co-author of the disputed paper, filed a claim of retaliation last year against Bauer for dropping her from his laboratory team immediately after she questioned Arbin's research in December 1995. Bauer denied the charge, saying that Bodkin's funding was running out and there were problems with her productivity. The US Office of Special Counsel, a watchdog agency that investigates such claims of retaliation against federal employees, is carrying out its own investigation.

Rex Dalton

Reform follows discontent at Tata institute

[NEW DELHI] A major shake-up has been approved of the Tata Institute of Fundamental Research in Mumbai (formerly Bombay), India's leading institute for basic studies in science and mathematics and the springboard for launching the country's nuclear programme in the 1940s.

This follows recommendations of a committee of international scientists headed by the British Nobel prizewinner Lord Porter, professor of chemistry at Imperial College, London, and a former president of the Royal Society. Unofficially, the reforms are also said to have been prompted by discontent among research staff about the way the institute has been run in recent years.

The panel's remit was to review the working of the institute during the past 25 years and to identify a possible strategy for the next ten years.

The institute, which is funded by the Department of Atomic Energy, was set up in 1946 by the late Indian nuclear physicist Homi Jehangir Bhabha. "We thought it was time to take stock of the accomplishments of the institute and plan its future activities," says Rajagopala Chidambaram, secretary of the department.

Although the authorities do not admit it, the review was also prompted by the simmering discontent among the institute's

academic staff who six years ago formed the Scientific Forum for the Tata Institute. This body told the review panel that the "rapid decline in the management of science in the institute" was a cause for concern, and that some of the problems "are so serious that they have caused the institute to remain stagnant".

In a letter submitted to the panel, the scientific forum said: "The shortsighted and self-centred policies of the authorities have made the scientists unhappy; there are serious difficulties in attracting new talent".

According to Tata scientists, the problems faced by the institute include subcritical research, poor management of research infrastructure, inadequate allocation of funds, unsatisfactory salary structure and lack of transparency and objectivity in personnel policies.

But Mambilikalathil Govind Kumar Menon, a former director of Tata and a member of its management council, says the problems are being exaggerated, adding that the review is long overdue.

The review panel's draft recommendations call for large-scale changes in the management, starting with the appointment of a new director from June. Chidambaram confirms that a search for a new director is taking place.

K. S. Jayaraman

Protests and promises grow in Russia as science spending falls

[MOSCOW] Russia's finance minister, Alexander Lifshits, promised scientists last week that he would speed up payment of the science budget, and expressed optimism that — unlike last year — it will be paid in full.

The promise followed growing discontent over the late payment of salaries, as well as the poor state of the science budget. President Boris Yeltsin has signed into law Russia's budget for 1997, already approved by both chambers of the Russian parliament, allocating 15,258 billion rubles (US\$2.6 billion) to research and development (R&D). Of this figure, 5,731 billion rubles will be spent on fundamental research and 9,526 billion rubles on the development of new technologies.

But the planned spending on R&D represents only 2.7 per cent of all budget expenditure — considerably less than the 4 per cent mandated in a law passed last August. Furthermore, there is growing concern among scientists that more than half this sum will make its way back to the government through taxation.

Proposed changes to the tax laws drawn up by the finance ministry would, if adopted, deprive scientists of privileges which are currently worth 8,000 billion rubles. Scientific organizations would be required to pay both value added tax and local land taxes, while all recipients of research grants would have to pay income tax on the money they receive.

"Under these conditions, foreign

organizations which support Russian science are likely to decide not to finance the bureaucracy, and thus to stop giving grants to Russian scientists," says Alexander Spirin, a member of the Russian Academy of Sciences.

Vladimir Kudryavtsev, a vice president of the academy and former director of its Institute of the State and Law, complains that the "new tax code had been prepared without consulting the scientific community, in strict secrecy, and in spite of the fact that the academy itself possesses hundreds of highly qualified specialist in its economics department."

The difficulties facing scientists are compounded by the fact that not all the money allocated to them actually arrives, while the distribution of funds between different fields of science is very uneven. Last year, for example, the Russian Space Agency received 97.3 per cent of what had been planned, a reflection of US pressure on Russia over its planned participation in the international space station; in contrast, the Russian Foundation for Fundamental Research received only 37 per cent.

With many scientists receiving their salaries at least a month late, a series of protest meetings has been held. These began in the academy's biological scientific centre at Pushchino near Moscow, where 3,000 of the 20,000 staff have demanded the resignation of the government and retirement of the president.

Such demands have been supported by

scientists in St Petersburg, Vladivostok and Moscow, where about 300 people picketed the White House, the main parliament building. Their representatives were met by Lifshits and Vladimir Fortov, chairman of the State Committee on Science and Technologies.

On behalf of the cabinet, Lifshits signed a promise that scientists will in future receive the same priority on salaries as teachers and service personnel. A schedule of monthly payments of the science budget was also approved: this month, scientists are to receive 950 billion rubles, and a further 750 billion rubles will be paid in each of the following three months — equivalent to a quarter of the annual budget.

A further meeting between members of the cabinet and representatives of the scientific community will be held in June to discuss plans for the second half of the year. Lifshits promised to find additional finance for science, in particular using foreign loans. He also expressed optimism that the remaining three-quarters of the science budget would be paid by the end of the year.

Meanwhile, Yeltsin has appointed Yuri Osipov, the president of the academy who was recently appointed to the cabinet, as a member of the Security Council. Another academy member, Boris Berezovsky, was recently made deputy chairman of the council. The 1,200 academy members are waiting to see whether the appointments will have any impact on their plight. **Carl Levitin**

Lab director exits bloody but unbowed

[WASHINGTON] Few modern research administrators have managed to personify their institution in quite the way that Nick Samios has come to represent the Brookhaven National Laboratory in New York state since his appointment as director there in 1982.

Then, Brookhaven had just been forced to abandon work on a giant particle accelerator called Isabelle, which had been intended to ensure the laboratory's future as a centre for particle physics research. Now, the Long Island laboratory is home to the National Synchrotron Light Source, a vastly popular source of X-ray, ultraviolet and infrared radiation.

Many smaller projects and centres have broadened the laboratory's scientific base. And the completion in 1999 of a brand new nuclear physics facility, the Relativistic Heavy Ion Collider, should assure Brookhaven a sparkling future.

Or so it appeared until the discovery in January of an underground plume of radioactive tritium emanating from Brookhaven's other large user facility, the

High-Flux Beam Reactor (see *Nature* 386, 3; 1997). The furor over the leak — which, as Samios ruefully observes, amounts to around 30 cubic millimetres of the hydrogen isotope — overshadowed the announcement on 7 March that he will quit as Brookhaven's director.

Tears were shed at the laboratory that morning, according to Brookhaven staff. They were probably provoked not by concern for Samios, who is well able to look after himself, but by exasperation inside the besieged laboratory as activists and local politicians — notably Senator Alfonse D'Amato (New York, Republican) — sought to blame Samios for the leak and take credit for his departure. "D'Amato will say anything he thinks will get him re-elected," observes one prominent physicist unconnected with Brookhaven.

Samios denies that he was forced out, saying that he had notified Associated Universities Inc., the contractor that runs Brookhaven, a year ago that he was intending to leave after 15 years in the job. Sources outside the laboratory confirm this. But they

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Samios: departure was agreed prior to recent controversy over leakage of tritium.

also point out that, without the furore over the leak, Samios would probably have offered to stay in place until a successor was found. Instead, he will step down at the end of next month.

Samios, who turned 65 last Friday, but looks and acts ten years younger, says that he did consider a resignation accepting blame for failing to find the leak earlier. But he rejected the idea, saying "It's not my style to quit, it's not true, and it wouldn't have solved very much."

The crisis that has engulfed Brookhaven since the leak was found has led Samios to reflect more widely on the public's understanding of science. "There's an anti-science mood in the world today," he says. "You see it all over; there is a distrust of science. Look at how worried people are about cloning." This mood, he says, "is stronger than it was five years ago. Science in general has to make more cogent arguments than it has in the past. We haven't communicated the importance and value of our work."

Samios shares the blunt, engaging and energetic manner that characterizes so many successful sons of New York city. A brilliant physicist whose discovery of the Omega Minus particle in the 1960s is considered by some colleagues to be worthy of a Nobel prize, he spurns intellectual aloofness and prides himself on being a man of action.

As chair of the American Physical Society's planning committee, for example Samios was a major behind-the-scenes force in the recent unified call by US scientific societies for more research funding (see *Nature* 384, 393; 1996).

Despite his deep involvement in science policy, Samios says he does not plan to work in Washington DC in the foreseeable future if he can help it. Having had little time for research in recent years, he says he is flattered to have already been invited to participate in scientific projects, but adds that it is "too early to comment" on what he might take up. Samios is supposed to complete a plan to clear up the tritium plume before he leaves. Running the laboratory has been "a wonderful responsibility", he says. "But not having that responsibility on my shoulders will be a great thing."

Brookhaven retains an interest in particle physics, and will be involved in the design of the Atlas detector for the Large Hadron Collider at CERN, the European Laboratory for Particle Physics in Switzerland. But its largest scientific interests now lie elsewhere. It needs a new director who can lead the laboratory beyond the Relativistic Heavy Ion Collider and into the next century.

In practice, this means a nuclear or condensed-matter physicist of exceptional political agility, ready and able to convince the people of Long Island that physics research can and must continue safely in their midst.

Colin Macilwain

A window of opportunity is opened for Italian reforms

[ROME] Italy is giving its research agencies a unique opportunity to reform their notoriously bureaucratic management structures – the bane of scientists for decades – and to develop more effective ways of allocating research funds.

The Italian parliament agreed last week that the government should have the power to modify existing laws, without further parliamentary approval, where this is needed to promote efficiency in public institutions. As a result, Luigi Berlinguer, the research minister, now has the right to "reorder and rationalize all efforts related to the promotion and support of scientific and technological research".

The new law states that, in making changes to the activities of research agencies, consideration should be given to the need to increase the institutional flexibility of researchers and their ability to move between research institutes, universities and industry.

The so-called Bassanini law, named after its parliamentary sponsor, will take effect for just one year. Committees set up by major research organizations — including the national research council (CNR), the National Agency for New Technology, Energy and the Environment (ENEA), the Italian Space Agency (ASI) and the National Institute for Nuclear Physics (INFN) — as well as many smaller organizations and institutes, have four months in which to draw up proposed changes.

The ministry of research will then revise the proposals and present them to parliament for debate.

Any changes will have to be in line with the government's strategic goals for research. Last December, the government set up a top-level committee of six ministers to develop a long-term research strategy based on clear priorities. These are being identified with the use of forecasting techniques and an analysis of Italy's research capacity and the needs of potential users of the research results.

A task force, headed by Giuseppe Tognon, under-secretary of state for research, is working with this committee to coordinate research and technology policies of the different ministries. "Previously, Italy's long-term plans have been no more than shopping lists, with little indication of priorities," says Alberto Silvani, a senior policy adviser to Berlinguer and a former researcher at the CNR Institute for Science Policy in Rome. Silvani says that the ministry wants to "concentrate, rather than scatter" its money, and plans to set up an assessment system to monitor the way in which research money is spent.



Berlinguer: powers to 'reorder' agencies

Few are prepared to speculate at this stage on likely changes to the research environment. But it is clear that the CNR will probably undergo major reform. The government is keen to see a clearer distinction between the roles of Italy's major research institutions. In particular it wants investigator-initiated research to be carried out exclusively in universities, which employ 60,000 professors, with the 3,000 researchers in CNR institutes carrying out strategic research.

There is also pressure from political and university quarters to remove the CNR's role in funding university research projects (see *Nature* 367, 398; 1994). Some have suggested that a new independent grant agency should be given this task, and that the CNR's central technology transfer support unit — which acts as a go-between for academics and industry — should be taken over by ENEA, which is Italy's principal applied research organization.

The Bassanini law will also permit reform of other research-related bodies such as the government's science and technology advisory committee (CNST). This was created by a 1989 law, but there have been no elections of representatives from the research community as no one has been able to define the electorate. Berlinguer would like the CNST to be restructured and to become fully active.

Luciano Maiani, the president of INFN — which is unlikely to face major changes — says that the most helpful thing the Bassanini law could do would be to increase the mobility of researchers between institutes and universities. "We need to change the way people work, rather than hold large strategic discussions," he says.

This could involve further efforts to abandon the centralized competitions for university positions (concorsi). Many would welcome such a move (see *Nature* 378, 228; 1995), but it has had little support in parliament, which traditionally has a high number of professors.

With only 12 months to overhaul their entire management structure, tensions are already beginning to run high among research administrators. Maiani admits that they face a difficult task ahead. But, echoing a widely held sentiment, he says that the Bassanini law is a great chance for Italy, "and it would be a disaster if the opportunity were to be lost".

Alison Abbott

Nuclear debate goes critical in Japan

Robert Triendl

The apparent incompetence of Japanese authorities in dealing with last week's fire at a nuclear plant is taking its toll of public confidence.

The Japanese government and the semi-public Power Reactor and Nuclear Fuel Development Corporation (PNC) have come in for severe criticism for their handling of an accident last week at a nuclear fuel recycling plant at Tokai village in Ibaraki Prefecture northeast of Tokyo.

The fire broke out on the morning of 11 March in a facility used for the 'bitumenization' of low-level radioactive waste. It was followed ten hours later by an explosion that blew out doors and windows.

Sloppiness in extinguishing the fire and in the subsequent clean-up appear to have resulted in volatile asphalt gases filling the room, eventually causing the explosion. Contrary to PNC's initial assertion that no radiation had escaped, low doses of radioactivity were released to the environment, and at least 37 PNC workers were exposed.

Japan's prime minister, Ryutaro Hashimoto, and the president of PNC apologized publicly for the slow response to the accident. But, reflecting growing public distrust in the government's nuclear policy, the governor of Ibaraki Prefecture visited both Hashimoto and the head of the Science and Technology Agency (STA) the following day, and urged a thorough investigation of the accident as well as of safety measures at all nuclear facilities in his prefecture.

"Contrary to all that has been said over the last year on the public release of information and crisis management, this accident shows very clearly that nothing has changed," says Minako Ogisa, a spokeswoman for the Fukui Prefecture Citizens' Forum Against Nuclear Power, the largest anti-nuclear group in the prefecture where Japan's experimental fast-breeder reactor Monju is located.

Long-term implications

A senior official at STA, the agency that oversees PNC, admits that, "to restore public confidence, a sound investigation, carried out publicly, is absolutely essential".

Most analysts agree that last week's accident had "at worst the character of a major accident at a chemical processing plant", and have been surprised by the harsh public reaction. But the long-term implications for the future of Japan's nuclear fuel-cycle policy could be substantial. "While the Monju accident [in December 1995] concerned the first

stage of the fuel cycle, the accident at Tokai village concerns the final stage," Ogisa points out.

Meanwhile, STA's final report on the liquid sodium leak at the Monju experimental fast-breeder reactor (see *Nature* 379, 196; 1996), released last month, has met considerable criticism from citizens' groups. "The ultimate reason for the accident remains untreated in the report," says Keiji Kobayashi, a professor of nuclear engineering at Kyoto University and a well known critic of Japan's fast-breeder programme.

"They do not deal with important deficiencies in PNC's approach to design and quality control," says Kobayashi. A critical assessment of the report by an expert group associated with the Fukui Prefecture citizens' forum is to be made public by the end of this month, says Kobayashi.

Earlier this year, a Fast-Breeder Reactor (FBR) Advisory Committee was set up by the Atomic Energy Commission and STA, at the request of the governors of Fukui, Niigata and Fukushima, the three prefectures where most Japanese nuclear power plants are located.

Opponents of the fast-breeder programme, and of nuclear energy in general, are relatively well represented on the committee and the agency is said to have consulted Jinzaburo Takagi, a prominent critic of Japanese nuclear policy, about the selection of committee members.

According to official sources, the FBR Advisory Committee aims at a broad reassessment of Japanese nuclear policy. An official at the agency points out that the committee is an "adventure", the outcome of which is "uncertain". He adds: "It's an entirely new situation we have no experience with. It will be necessary to proceed in an *ad hoc* fashion, without invoking grand strategies."

But some observers dismiss this as mere tactical manoeuvring. "It's all about window dressing. What they want is to disperse criticism and pave the way for reopening Monju," says Kobayashi.

Others admit that critics have now at least found their place inside the government. "It is true that critical observers are now participating in various expert advisory bodies related to nuclear issues," says Hitoshi Yoshioka, a professor of sociology at the University of Kyushu and the leader of the anti-nuclear faction on the FBR Advisory Com-

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The morning after: glass litters the recycling plant after explosion blew out windows.

mittee. But he adds that the actual role of the committee remains rather vague.

Some analysts argue that the setting up of the FBR Advisory Committee may be a sign of desperation, and even crisis, at STA. In a mood of doom about STA's nuclear fuel-cycle programme, an old rivalry between the agency and the Ministry of International Trade and Industry over the control of Japanese nuclear politics could regain momentum. Takagi says: "We have been struggling with STA bureaucrats for years. Now that we have started to get along with them, we recognize that our most important opponent is elsewhere."

Death knell for Monju

After last week's accident, critics of Japan's fast-breeder programme are increasingly confident that Monju will never go on line. "Monju is a dead end," says Ogisa. Yoshioka adds: "This is something Japan is good at. Everything comes ten years too late. The start of the Japanese nuclear energy programme was ten years late. Japan's FBR programme took off with a ten-year delay, and now it will be abandoned ten years too late."

Although it may be too early to speculate about the ramifications of last week's accident for Japanese nuclear policy, there appears to be a widespread consensus that the accident may contribute to a considerable slowdown of plans to burn mixed plutonium-uranium fuel (MOX) in ordinary light-water reactors.

This plan, known as the 'plutothermal' programme, was approved by the Japanese government in early February. But reactors still have to be relicensed by local authorities to burn MOX (see *Nature* 385, 757; 1997). And after last week's accident they are not likely to give their approval soon. □

The author is an expert on Japanese science policy currently based in Paris.

US fusion machine gets the green light despite court order

[WASHINGTON] The US Department of Energy last week gave the final go-ahead for construction of the National Ignition Facility, the proposed inertial confinement fusion machine at the Lawrence Livermore National Laboratory in California. The decision was made despite a court order, based on a suit filed by an environmentalist group, prohibiting the department from using the advice of a National Research Council panel which had just reported that the facility is likely to achieve its technical goals.

According to officials, the department did not need the report to proceed. Construction of the \$1.2 billion, 192-laser machine is likely to start in April. The facility is intended primarily to help physicists to ensure the reliability of US nuclear weapons without resort to testing, but will also study the feasibility of inertial confinement fusion as an energy source.

NIST boss departs for private sector

[WASHINGTON] Arati Prabhakar, director of the US National Institute of Standards and Technology (NIST), is leaving the Clinton

administration to take up a position in the private sector. Prabhakar, who is 38, was an energetic champion of the technology programmes at NIST which President Bill Clinton sought to expand during his first term. She will join Raychem in Menlo Park, California, as chief technology officer. Robert Hebner, who has been acting as director of NIST since Prabhakar went on maternity leave in January, will continue in the post until a successor is chosen and confirmed by the US Senate.

Senators revive plan for research levy

[WASHINGTON] Two US senators, Tom Harkin (Democrat, Iowa) and Arlen Specter (Republican, Pennsylvania), have reintroduced a proposal that would require health insurance companies to put 1 per cent of revenues — about \$6 billion a year — in a trust fund to support research at the National Institutes of Health.

Harkin said that the measure “has an excellent opportunity” to succeed if it attracts enough grass-roots support. But June O'Neill, director of the congressional Budget Office, said the idea of a trust fund separate from the main federal budget is “worrisome” to advocates of fiscal restraint, as so many special interest groups think that they deserve one.

Plea to drop charges in French HIV scandal

[PARIS] Laurent Fabius, the former French prime minister who is facing charges of collusion in poisoning in the HIV-contaminated blood affair (see *Nature* 364, 269; 1994), “acted as quickly as possible” at the time, examining magistrates were told last week.

Jean-François Burgelin, attorney general of the Court of Justice of the Republic, asked for the dismissal of the cases against Fabius and the two other former ministers charged in the affair — Edmond Hervé, former secretary of state for health, and Georgina Dufoix, former social affairs minister. Burgelin's brief to the magistrates rejects the criminal responsibility of Hervé and Dufoix but is still highly critical of them. It complains of inaction by them and their offices, and of “blindness” and “apathy” on the part of Hervé. The examining magistrates of the Commission d'Instruction have since announced that they will continue their investigation of the former ministers.

Science policy units club together

[PARIS] Fourteen science policy research units from 11 European countries have formed a consortium, the European Science and Technology Observatory Network. Members



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include the French Observatoire des Sciences et Techniques and the UK Science Policy Research Unit at the University of Sussex. The consortium will carry out studies in key areas involving technology, the economy and society.

The group will be administered by an executive board chaired by the Institute for Prospective Technological Studies, one of the European Commission's joint research centres, and coordinated by the VDI Technology Centre in Germany.

Scottish wave power set for relaunch

[PARIS] The Scottish company Applied Research & Technology is planning to relaunch a successor to Osprey 1, a 2-MW prototype wave generator that was destroyed by a massive sea swell during its installation off the north coast of Scotland in summer 1995 (see *Nature* 377, 6; 1995). The accident occurred after a sudden change in weather conditions which resulted in erosion of the generator's sandy foundations before engineers had time to lay down stabilizing mats. To avoid a recurrence, Osprey 2 has been fitted with a new ballast system. According to Allan Thomson, managing director of the company, this will reduce the time it should take to install the machine on the seabed from five days to 24 hours.

Rosy prospects for British biotech

[LONDON] British biotechnology is booming, thanks largely to increased government support, changes in the pharmaceutical industry that favour out-sourcing and 'vertical integration', and improved access to finance, according to a report published last week by management consultancy Arthur Andersen. The report, *UK Biotech '97 — Making the Right Moves*, says that revenues for all companies in the biotechnology sector increased from £470 million (US\$750 million) in 1992–93 to £702 million in 1995–96, and predicts that this will more than double by 1997–98. As a result of this growth, ten UK companies would rank in size among the top 50 such companies in the United States. Three years ago, only four UK companies were of such standing.

Japanese farmer sues over *E.coli* 'slur'

[TOKYO] A radish sprout farmer in Osaka Prefecture last week filed suit against the Japanese government demanding ¥53 million (US\$425,000) in compensation for the announcement last summer by the health ministry that white radish sprouts (*kaiware daikon*) from his farm were the likely cause of the mass infection of schoolchildren in Sakai

City near Osaka with the O157 strain of *Escherichia coli* (see *Nature* 382, 567; 1996).

Hajime Minamino, head of the Minamino Farm in Habikino, claims that the ministry's announcement had no scientific basis. No bacteria of the O157 strain were ever found at the farm, despite inspections by the government, and he says the announcement caused the farm to lose ¥30 million in income compared with the previous year.

India offers help to ex-Soviet scientists

[NEW DELHI] The Indian government is to open its leading research institutions to scientists from the nations of the former Soviet Union to assist them during a critical financial period. Researchers from these countries, especially younger scientists, will be invited to work in the place of their choice for up to two years.

Under an existing arrangement, several scientists from former Soviet countries have already made three visits to India. "We now want to go beyond this," says Valangiman Ramamurthy, secretary to the Department of Science and Technology.

correction

Our picture on page 8 of the issue of 6 March was incorrectly labelled. The individual on the left of the picture is Ron James, managing director of PPL Therapeutics.

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Uppsala, Sweden. (And the rest of the world)

Smaller grants for more Canadians?

Sir — We often hear that the major problem with scientific and medical research in Canada is the insufficient level of government funding. The biggest sources of research funding for universities are the three federal research funding councils, the Natural Sciences and Engineering Research Council (NSERC), the Medical Research Council (MRC) and the Social Sciences and Humanities Research Council (SSHRC).

Are these agencies underfunded? According to the admission of the councils themselves, many researchers who receive evaluations of excellence do not receive funding because the granting agencies run out of money before getting to their particular proposal. Deciding which of two excellent proposals will or will not be funded is never easy. Committees must therefore make decisions on the basis of differences that are largely irrelevant to the potential contribution of a particular proposed research project.

There are two possible solutions. The first would be to make it possible for all excellent programmes to get funding at the level requested by the applicants. This is clearly impossible in the current political state of restraint. As the pie is limited, perhaps we should be asking whether we are managing the pie and cutting the pieces fairly, to which the answer is a resounding no.

According to the latest report from MRC, it approves about 600 new grants a year, but is turning down another 350 proposals that meet its standards of

excellence. The question then boils down to the following: If you have $600 + 350 = 950$ proposals a year which satisfy MRC standards of excellence, what stops MRC from funding them all? In a zero-sum game, of course, this will mean that the average grant size should drop to $600/950 = 63\%$ of its present average. But then there would be no excellent researchers without funds.

A reduction to 63% may appear severe to the already funded researcher. But for the researcher who gets nothing now, that amount would be considered a great boon. Tough as it appears, this simple change would allow all "excellent" researchers to be funded and to implement at least their main research priorities. Furthermore, because more scarcity will undoubtedly result in a much more careful setting of priorities, the overall quality of research is likely to increase, not decrease, and all "excellent" proposals will be funded on at least some level.

This is the first thing MRC (and likewise NSERC and SSHRC), should do before asking the government for more money. Those at present involved in the campaign for more money for the funding councils should first of all ask the councils' presidents what are the guarantees that this "extra" money will be used to fund those at present unfunded, instead of being distributed among those who are already well funded. The present structure of the funding councils provides no such guarantees.

The existing composition of funding

councils is such that the key members of the grant selection panels are grant recipients themselves. All assurances to the contrary notwithstanding, this constitutes a fundamental conflict of interest. It is not then surprising that the distribution of research grants follows the infamous Matthew principle ("the rich get richer, the poor get poorer"). In other words, those who decide how to cut the pie are the same people who want to ensure they get their own piece first.

This is where the most urgent change is needed. We need restoration of the fundamental 'arm's length' principle that those who distribute the research funds should not themselves be the beneficiaries of the funding system (grant recipients). This would be a first step towards more productive and efficient use of Canadian research dollars. Only after that will we be able to decide if we really have genuine research underfunding, or rather a case of poor management of available resources.

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Brussels backs research

Sir — I should like to comment on your leading article "Science and technology deserve better from Brussels" (*Nature* 385, 661; 1997) which gives an account of the European Commission's recent proposals for the European Union's fifth Framework programme for research.

You state that the commission has not achieved the concentration on a few areas which it presented several months ago as its objective. In fact, what the commission seems actually to have attempted, with some success, is to strike a balance between concentration on a selected number of objectives essential for a significant impact in economic and social terms and support for sufficient collaboration in some fundamental fields to ensure the continued development of European scientific and technological excellence. This double objective explains the presence in the proposal of a larger number of topics than there might

otherwise have been.

Will the steps towards concentration taken so far be translated into reality? The European Union's decision-making system for research, which involves the unanimous adoption of the Framework programme by the member states jointly with the European Parliament, is far from ideal. Having been commissioner for research, I know how difficult it is to avoid spreading our research efforts over a multiplicity of subjects corresponding to the preferences of the member states and various sectoral interests.

The present research commissioner, Edith Cresson, appears to be firmly resolved to defend the principle of real concentration. Stating, as you do, that the fight is already lost, seems to me to be, at the very least, premature. It is not unreasonable to hope that the Framework programme, when it is finally adopted, will correspond to the ideas already presented. The commission has already announced that it will set up an

appropriate management system for its implementation. Saying, as you do, that the Framework programme will be impossible to manage is inappropriate.

Finally, your explanation of the root cause of the supposed weakness of the commission's proposals shocked me. I am astonished that you should call into question the commissioner's interest in research. The significant role which, according to your own account, the fifth Framework programme assigns to fundamental research bears witness to the opposite opinion, and shows that Cresson has fully appreciated the importance for society of the pursuit of knowledge.

Antonio Ruberti

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Rome, Italy*

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The first elementary particle

The electron is 100 years old this year. Of all the elementary particles, it is by far the most familiar, useful and venerable. But is it elementary? And what other particles are elementary?

Steven Weinberg

There was a double irony in the discovery of the electron by J. J. Thomson 100 years ago. For one thing, the discovery was a triumph more of theoretical preconceptions than of experimental virtuosity. Thomson's reputation when he was called to the Cavendish professorship in experimental physics was largely based on his mathematical work; one of his assistants at the Cavendish Laboratory recalled later that "J. J. was very awkward with his fingers, and I found it necessary not to encourage him to handle the instruments". His crucial 1897 measurement of the mass-to-charge ratio of the particle making up cathode rays was not as accurate as the measurements done at about the same time by Walter Kaufmann at Berlin. But Thomson had been raised in the atomistic intellectual tradition of Newton, Prout and Dalton, and found it natural to leap from the observation that the mass-to-charge ratio of the cathode-ray particle was both small and independent of the material of the cathode from which it was emitted, to the conclusion that this particle was the basic constituent of atoms, "the substance from which the chemical elements are built up".

Perhaps Thomson's best achievement as an experimentalist was his demonstration two years later that the particles emitted from metal surfaces in the photoelectric and thermoelectric effects have the same ratio of mass to charge as the particle of the cathode rays. This supported his claim that these are all the same particle. It was at about this time that physicists began to call this particle by a name borrowed from the theory of electrolysis: the electron.

Thomson also carried out a crude measurement of the electric charge of the electron, from which one could immediately infer the charges of ions, atoms that have gained or lost one or more electrons. Together with earlier measurements of the mass-to-charge ratios of various ions in electrolysis, this gave values for the masses of ions and hence also of atoms, in agreement with those inferred by other means. This is a second and greater irony: the discovery of the electron helped to establish the existence of atoms, but as electrons could be ripped out of atoms, this discovery also showed that atoms were not what they had always been thought to be: they are not the indivisible constituents of matter. As we would say today, they are not elementary particles.

So what are the elementary particles? When atomic nuclei were discovered in Ernest Rutherford's laboratory in 1911, it

J. J. Thomson: discovering "the basic constituent of atoms".

was assumed that they were not elementary, partly because it was known that some radioactive nuclei emit electrons and other particles, and also because nuclear charges and masses could all be explained by assuming that nuclei are composed of two types of elementary particle: light, negatively charged electrons and heavy, positively charged protons.

In the 1920s, the idea that all matter consists of just two types of elementary particle became pervasive and resilient in a way that is difficult to understand today. For instance, when the electrically neutral neutron was discovered by James Chadwick in 1932, it was generally assumed that it is a composite of one proton and one electron. In his paper announcing the discovery, Chadwick offered the opinion: "It is, of course, possible to suppose that the neutron is an elementary particle. This view has little to recommend it at present, except the possibility of explaining the statistics of such nuclei as ^{14}N ." (One might have thought this was a pretty good

"The difference between elementary and composite particles has... basically disappeared. And that is no doubt the most important experimental discovery of the last 50 years."

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reason: by "statistics" Chadwick was referring to evidence from molecular spectra that ruled out the possibility that the ^{14}N nucleus consists of just protons and electrons, whether or not some of them are bound together as neutrons.) It was the discovery of the charge independence of nuclear forces in 1936 that showed clearly that neutrons and protons have to be treated in the same way; if protons are elementary, then neutrons must be elementary too. Today, in speaking of protons and neutrons, we often lump them together as nucleons.

This was just the beginning of a great increase in the roster of so-called elementary particles. The first new particle to be discovered was the positron, the antiparticle of the electron. In Paul Dirac's 1928–30 theory of the electron, the positron appears as a hole in a sea of negative-energy electrons, but today this interpretation is obsolete and the positron is regarded as a particle in its own right. The muon (a sort of heavy electron) was added to the list in 1937 (though its nature was not understood until later), and strongly interacting particles called pions, kaons and hyperons were discovered in the late 1940s. The weakly interacting particle called the neutrino had been proposed in 1930 but was not detected until 1955. Then, in the late 1950s, the use of particle accelerators and bubble chambers began to reveal a great variety of new strongly interacting particles, including heavier cousins of the pions, kaons, nucleons and hyperons.

On the principle that, even if there are

more than two types of elementary particle, there really should not be many of them, theorists speculated that most of these particles are composites of a few types of elementary particle. But how could one tell whether the electron or any other of these particles is among the ones that are elementary? As soon as this question was asked, it was clear that the old answer — that particles are elementary if you can't knock anything out of them — was inadequate. Electrons and positrons are created in the collisions of electrons with each other or with atomic nuclei, but we can't think of them as knocked out of the electron. Pions come out when protons collide with each other, and protons and antiprotons come out when pions collide with each other, so which is a composite of which?

In the 1950s, this dilemma was turned by some theorists into a point of principle, known as 'nuclear democracy', which held that any particle may be considered to be a bound state of any other particles, as long as conservation laws are respected. This view was reflected decades later in a 1975 talk to the German Physical Society by Werner Heisenberg, who reminisced that: "In the experiments of the fifties and sixties... many new particles were discovered with long and short lives, and no unambiguous answer could be given any longer to the question about what these particles consisted of, since this question no longer has a rational meaning. A proton, for example, could be made up of neutron and pion, or Lambda-hyperon and kaon, or out of two nucleons and an anti-nucleon; it would be simplest of all to say that a proton just consists of continuous matter, and all these statements are equally correct or equally false. The difference between elementary and composite particles has thus basically disappeared. And that is no doubt the most important experimental discovery of the last 50 years."

Elementary field

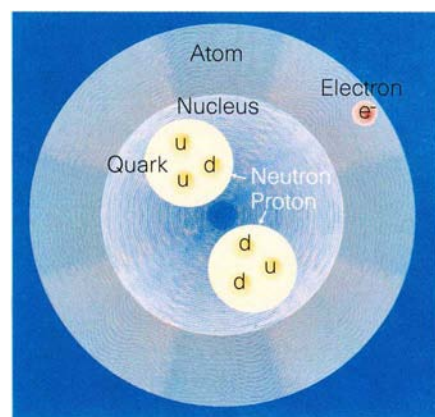
Long before Heisenberg reached this rather exaggerated judgement, a different sort of definition of elementary particle had become widespread. From the perspective of quantum field theory, as developed by Heisenberg, Pauli and others in the period 1926–34, the basic ingredients of nature are not particles but fields; the photon is a bundle of energy of the electromagnetic field, and the electron is a bundle of energy of a different field, called the electron field. It is natural to define an elementary particle as one whose field appears in the fundamental field equations. It doesn't matter if the particle is heavy or light, stable or unstable — if its field appears in the field equations it is elementary; if not, not.

This is a fine definition if one knows the field equations, but for a long while physicists didn't, except in the one case of quantum electrodynamics, the theory of electrons, positrons and photons. A fair amount of

theoretical work in the 1950s and 1960s went into trying to find some objective way of telling whether a given particle type is elementary or composite, when the underlying theory is not known. This turned out to be possible in certain circumstances in nonrelativistic quantum mechanics; one can show for instance that the deuterium nucleus spends most of its time as a bound state of a proton and neutron. This was not exactly a thrilling achievement — everyone had always assumed that the deuteron is a bound state — but this demonstration had the virtue of relying only on nonrelativistic quantum mechanics and low-energy neutron–proton scattering data, without any specific assumptions about the nuclear force or about what happens at high energy. Unfortunately, arguments of this sort cannot be extended to electrons or the other particles encountered in elementary particle physics.

The lack of any purely empirical way of distinguishing composite and elementary particles does not mean that this distinction is not useful. In the 1970s, the distinction between elementary and composite particles seemed to become much clearer, with the general acceptance of a quantum field theory of elementary particles known as the Standard Model. It describes fields of quarks (the constituents of nucleons, hyperons, pions and so on); of leptons (the electron, muon and the more recently discovered tauon; together with associated types of neutrinos) and of the photon and 11 similar particles — the eight gluons which produce the strong forces that hold quarks together in the nucleons; and the W^+ , W^- and Z^0 particles, which produce the weak forces responsible for radioactive processes involving neutrinos.

So, at least for the present, these are the elementary particles: the quarks, leptons and the photon and its siblings. The proton and neutron and all the hundreds of strongly interacting particles discovered after the Second World War are not elementary after all;



The deuterium atom consists of an electron wave surrounding a nucleus, which consists of a proton and a neutron, which consist of u and d quarks.

they are composites of quarks and gluons — not because we can knock quarks and gluons out of them, which is believed to be impossible, but because that is the way they appear in the theory.

The one uncertain aspect of the Standard Model is the mechanism that gives the elementary particles their masses. In the simplest version of the model, these masses depend on constants that specify the strength of the interaction of the various elementary particles with a new kind of field that pervades the Universe, but these constants are just free parameters of the theory. Wanting any better ideas, it would be most natural to expect that all these constants are roughly equal to the constant that specifies the strength of the interaction of the electron and other charged particles with the electromagnetic field — that is, to the charge of the electron. In this case all the elementary particles would have masses that are either zero or roughly equal to the masses of the W^+ , W^- and Z^0 particles. This is far from the case — almost all the elementary particles are much lighter than the W^+ , W^- and Z^0 particles. In particular, the lightest of all the massive elementary particles

Table 1 **Fermions (spin = 1/2)**

Leptons			Quarks		
Flavour	Mass (GeV/c ²)	Electric charge	Flavour	Mass (GeV/c ²)	Electric charge
ν_e electron neutrino	$<7 \times 10^{-9}$	0	u up	0.005	$2/3$
e electron	0.000511	-1	d down	0.01	$-1/3$
ν_μ muon neutrino	<0.0003	0	c charm	1.5	$2/3$
μ muon	0.106	-1	s strange	0.2	$-1/3$
ν_τ tau neutrino	<0.03	0	t top	170	$2/3$
τ tau	1.7771	-1	b bottom	4.7	$-1/3$

Table 2 **Bosons (spin = 1)**

Unified electroweak	Mass (GeV/c ²)	Electric charge	Strong or colour	Mass (GeV/c ²)	Electric charge
γ photon	0	0	g gluon	0	0
W^-	80.22	-1			
W^+	80.22	+1			
Z^0	91.187	0			

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS UNAVAILABLE FOR COPYRIGHT REASONS

By studying the cathode rays in a Crookes tube from which gas had been evacuated, Thomson concluded that they are produced by an electric current of particles.

is the electron; its mass is 180,000 times smaller than the Z^0 mass. No one knows where a number this size comes from. From this point of view, the electron is the most mysterious of all the elementary particles.

Through all this series of discoveries, the electron has kept its status as an elementary particle, but it is now known to be just one of three electrically charged leptons. The fact that electrons are important in ordinary life whereas muons and taus are not just reflects the fact that it is the lightest of the three, and hence stable. The extra energy in the muon and tauon's masses can be released when these particles decay into electrons and neutrinos, so they do decay and hence are not present in the matter around us, whereas the decay of the electron into a muon or tauon would violate the conservation of energy. But in principle we can also turn an electron into a muon or a tauon by hitting it with a high-energy neutrino of the right sort. There is no sign that electrons play a more fundamental role in the laws of physics than do the other leptons or the quarks.

The successes of quantum field theory and the Standard Model cast an interesting light on early conceptions of the electron. In his celebrated 1928 work on relativistic quantum mechanics, Dirac had concluded that electrons must have a certain angular momentum, or spin, equal to one-half in the units that are natural in atomic physics. The fact that the electron has spin one-half had been discovered a few years earlier, and still seemed quite mysterious, so this prediction was treated as a great success.

It is not always recognized even today that, although Dirac's theory was a huge step forward in our understanding of the electron, his argument about the spin of the electron was incorrect. Dirac's analysis did not rest on any special property of the electron, but only assumed that it is an elementary particle, which Dirac tacitly supposed meant that it must be described by a relativistic generalization of the non-relativistic wave mechanics of Schroedinger.

As Dirac pointed out, this would indeed run into problems with negative probabilities if an elementary particle had any spin other than one-half, so Dirac's reasoning would lead to the conclusion that *all* elemen-

tary particles must have spin one-half. But we now know of other particles like the W^+ , W^- , and Z^0 (as well as massless particles like the photon and gluons) that seem to be every bit as elementary as the electron, but have spin one rather than spin one-half. The trouble with Dirac's reasoning is that relativistic quantum mechanics does not need to be formulated as a relativistic generalization of wave mechanics; quantum field theory offers a more general approach, which allows for elementary particles of any spin.

Final answer

The success of the Standard Model might have been the end of the story of identifying the elementary particles, but since the late 1970s our understanding of quantum field theory has taken another turn. We have come to understand that particles may be described at sufficiently low energies by fields appearing in so-called effective quantum field theories, whether or not these particles are truly elementary. For instance, even though nucleon and pion fields do not appear in the Standard Model, we can calculate the rates for processes involving low-energy pions and nucleons by using an effective quantum field theory of pion and nucleon fields rather than of quark and gluon fields. In this field theory, pions and nucleons appear as elementary, though nuclei do not. When we use a field theory in this way, we are simply invoking the general principles of relativistic quantum theories, together with any relevant symmetries; we are not really making any assumption about the fundamental structures of physics.

From this point of view, we are entitled only to say that the quarks and gluons as well as the photon, W^+ , W^- , Z^0 , and the electron and other leptons are more elementary than nucleons and pions, because their fields appear in a theory, the Standard Model, that applies over a much wider range of energies than the effective field theory that describes nucleons and pions at low energy. We cannot reach any final conclusion about the elementarity of the quarks and gluons or even the electrons themselves. The Standard Model itself is probably only an effective quantum field theory, which serves as an approximation to some more fundamental theory

whose details would be revealed at energies much higher than those available in modern accelerators, and which may not involve quark or lepton fields at all.

It is possible, for instance, that the quarks and electrons and other particles of the Standard Model are themselves composites of more elementary particles. One way to tell is to look at the sizes of these particles, as measured for instance by the strengths of the magnetic fields produced by their spins. Having a non-zero size is not in itself a sign that a particle is not elementary. For instance, in quantum electrodynamics (which is today part of the Standard Model) the electron appears as an elementary particle, but it is nevertheless surrounded with a cloud of short-lived photons and electron-positron pairs, which give it a finite extension. The important thing is whether the measured effects of the finite size of the electron are equal to those calculated in quantum electrodynamics. So far, it seems they are. Experiments show that the strength of the magnetic field of the electron is 1.001159652188(4) in natural atomic units, while quantum electrodynamics gives a value 1.00115965214(3). This spectacular agreement shows that, if electrons are composite particles, then the energies involved in their binding must be very much larger than any energies probed in today's high-energy physics experiments.

We may not be able to give a final answer to the question of which particles are elementary until we have a final theory of force and matter. When we have such a theory, we may find that the elementary structures of physics are not particles at all. Many theorists think that the fundamental theory is something like a superstring theory, in which the electron, quarks and so on are just different low-frequency modes of vibration of the strings. From the point of view of superstring theory, fields like the electromagnetic field and the electron field are not fundamental, but appear only in an approximate description of phenomena, valid at energies that are too low to excite the higher modes of vibration of the string. It seems impossible in principle to identify one set of strings as truly elementary because, as recently realized, various different string theories with different types of string are actually equivalent.

There is a lesson in all this. The task of physics is not to answer a set of fixed questions about nature, such as deciding which particles are elementary. We do not know in advance what are the right questions to ask, and we often do not find out until we are close to an answer. □

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* Parts of this article are adapted from an essay "What is an Elementary Particle?" in *The Beamline* 27, 17–21 (1997).

Real engines of creation

Steven M. Block

Some enzyme complexes function literally as machines, and come equipped with springs, levers and even rotary joints. Clever biophysical techniques allow new approaches to macromolecular mechanics.

"Consider the lilies of the field, how they grow; they toil not, neither do they spin."

Matthew vi:28

The crystal structure of H^+ -ATP synthase — the enzyme that produces most of the ATP in our bodies — has a tripartite symmetry resembling the petals of a flower, somewhat reminiscent of a French fleur-de-lis. But this enzyme is no lily of the field: not only does it toil, it also spins! On page 299 of this issue, Noji and co-workers¹ describe an elegant experiment in which the normal flow of energy through the enzyme is reversed by supplying ATP. The result is the unidirectional rotation of individual complexes through hundreds of revolutions at speeds of up to around 4 Hz. This remarkable demonstration shows unequivocally that H^+ -ATP synthase is precisely what many have believed for some time: the world's smallest rotary motor.

Until now, that title was held by the flagellar motor, which is responsible for turning the filaments of swimming bacteria^{2,3}. Bacterial motors are impressive devices indeed — in some species, they turn at speeds in excess of 1,000 Hz, and they can propel cells at a rate of $100 \mu\text{m s}^{-1}$ or more^{2,4}. Transposed into our own dimensions, if cars could drive at equivalent scale speeds, they would break the sound barrier. Flagellar motors are formed from many protein components, and they are each about the size of a virus (around 40 nm across by 60 nm high). In contrast, the catalytic region of H^+ -ATP synthase is made up of a mere nine protein subunits with the stoichiometry $3\alpha:3\beta:1\gamma:1\delta:1\epsilon$, approximating a flattened sphere, just 10 nm across by 8 nm high, subtending less than one-tenth the volume of a flagellar motor⁵. But both H^+ -ATP synthase and the flagellar motors are macromolecular engines, and they run off the same fuel source: the protonmotive force.

In 1978, Peter Mitchell received the Nobel prize in chemistry for his chemiosmotic hypothesis⁶, which explains how the energy that is derived from respiration is ultimately coupled to ATP synthesis, flagellar rotation in bacteria and other fundamental processes. Briefly, cells use energy from the transport of electrons through the respiratory chain to pump protons from the inside of mitochondria (or the bacterial cell) to the outside. The

result is a transmembrane proton gradient consisting of two components: an electrical gradient and a pH gradient (Nernst potential), either of which can be tapped interchangeably for work. In a respiring bacterium, some 200 mV of electrical potential are represented by the protonmotive force.

Protein-based machines have evolved to use this powerful source of energy, and the premier example is H^+ -ATP synthase (which is also known as the F_1F_0 ATPase). It is a mushroom-shaped enzyme, consisting of two parts that are readily separated — the F_0 portion (stalk) is a proton channel that spans the membrane, and the F_1 part (button) is a cytoplasmic domain containing the active (catalytic) site(s) (Fig. 1). In energized mem-

branes, intact F_1F_0 complexes allow protons to travel down the electrochemical gradient and enter the mitochondrion (or bacterial cell). In some fashion that remains mysterious to this day, energy that is lost by these protons is used to synthesize ATP from ADP bound to the F_1 subunit. Isolated F_1 fragments, in contrast, work 'backwards' as ATPases, and these have long served as model systems to study the catalytic function of H^+ -ATP synthase.

Enter Paul Boyer. In the mid-1970s, Boyer and colleagues showed that the proton gradient is involved specifically in promoting the release of ATP from ATP synthase, and that all three of the F_1 catalytic sites (empty, ADP-binding and ATP-binding) are highly cooperative. This led them to propose the so-called 'binding-change mechanism'⁷, whereby the ADP and inorganic phosphate (P_i) that are bound at one catalytic site directly promote the release of ATP from an adjacent site — presumably by some allosteric effect. By studying the statistical distribution of ^{18}O exchange into all 'isotopomers' of ATP and P_i formed by catalysis, Boyer and David Hackney concluded that all three of the participating sites were biochemically equivalent^{8,9}.

When DNA sequence information for the F_1 -ATPase became available, all three α -subunits were found to have an identical sequence, as did all three β -subunits. How-

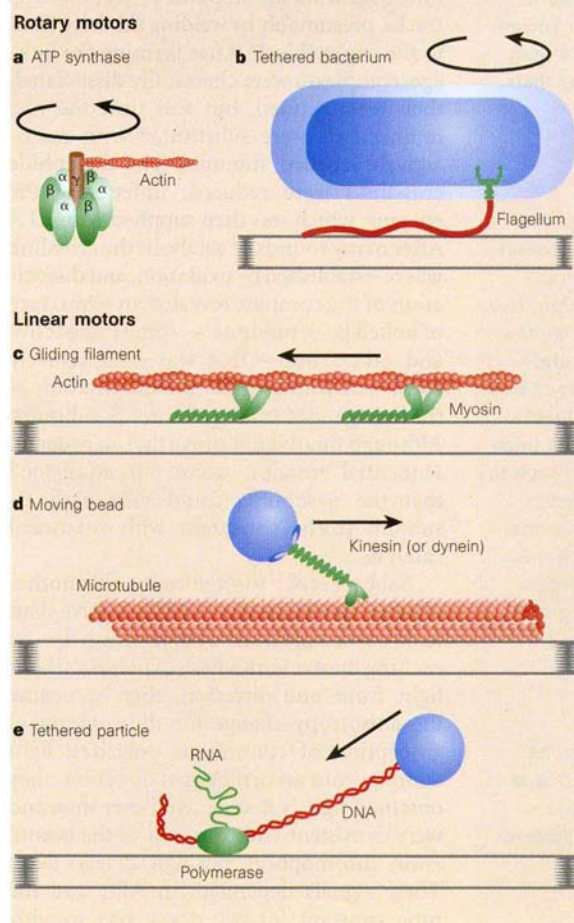


Figure 1 Motility assays for studying molecular motors^{3,14,15}. **a**, The F_1 rotation assay described by Noji *et al.*¹: an actin filament is attached to the F_1 subunit of H^+ -ATP synthase, and the filament is driven in a circular motion by the enzyme. **b**, The tethered cell assay: torque generated by the flagellar motor causes the bacterial cell body to rotate about the point of attachment of its flagellum to a surface. **c**, The gliding filament assay: a surface coated with stationary motors propels filaments over itself. Actin is shown here moved by myosin, but the assay works equally well with kinesin or dynein and microtubules. **d**, The moving bead assay: motors are attached to microscopic spheres, which are propelled along a microtubule. **e**, The tethered particle assay for RNA polymerase: a stationary enzyme complex bound to a surface moves processively along DNA, drawing a microscopic sphere towards itself. Motors shown in **a** and **b** are rotary motors, and **c–e** are linear motors. Motion takes place in the direction indicated by the arrows, and diagrams are not drawn to scale.

NATURE

100 YEARS AGO

It is only sixty years ago since George Catlin wrote his "North American Indians," and graphically described the vast herds of bison, numbering millions of individuals, travelling for days together across the rolling prairies; yet we have seen these disappear, like the aborigines, and their places usurped by the "cow-boy," and by countless herds of domestic cattle. If we could only wind the clock of Time still further backwards, and make him disclose, with moving-photographic-vividness, some of those earlier Mesozoic scenes on the American continent, or even in our own little island, for that matter, we should find, not herds of bison, but far other cattle, though some wore horns, and were big and ugly enough in all conscience; yet they were mostly harmless, and herbivorous in diet, but belonging to patterns now entirely obsolete, like the old "brown-bess" of our grandfathers' days, only more so. ... One of the most curious points about these mediæval animals is, that they make their earliest appearance in the Triassic period, and were first known in North America some sixty years ago, not by their bones or teeth, but by their footprints. — "Dinosaurs."

From *Nature* 18 March 1897.

50 YEARS AGO

The sudden widespread interest in the Antarctic, aroused by the announcement that no less than twelve nations are organising expeditions to that region, has resulted in a spate of articles which has been chiefly remarkable for inaccurate rumours and speculations. Supposed rich deposits of uranium and other valuable minerals have been depicted as the prize for the winners of an international race to stake out territorial claims. ... What is now happening is the realization on the part of all concerned that the economic development of this frozen continent is technically within sight — providing there is anything there which is worth the trouble of development.

From *Nature* 22 March 1947.

Many more extracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact David Plant (e-mail: subscriptions@nature.com).

ever, the sequence of the lone γ -subunit did not show any tripartite character and yet all three of the enzyme sites needed to interact with it to function. These facts led Boyer to propose a radical idea; namely that the binding-change mechanism did not correspond to allosteric activation of three sites in sequence. Instead, by analogy with the flagellar motor, the α - and β -subunits literally turned with respect to an axially located γ -subunit, bringing each of the three sites into play, one after the other¹⁰. This model was dubbed 'rotational catalysis'.

At first, few people believed this idea. Perhaps the γ -subunit had greater symmetry (or mobility) than imagined. Perhaps there was an allosteric explanation. Perhaps the enzyme just oscillated back and forth. But when the crystal structure of the F_1 -ATPase was eventually solved⁵, it looked every bit like a three-piston rotary motor, with a hexagonal ring of α - β pairs surrounding a drive shaft made up of the γ -subunit (Fig. 1 of Noji *et al.*, page 300). Armed with a detailed knowledge of the structure, two groups soon weighed in with experimental data in support of rotational catalysis.

Duncan *et al.*¹¹ genetically engineered a cysteine residue on the β -subunit, at a location near to a corresponding cysteine residue of the γ -subunit. This allowed them to form a reversible disulphide crosslink between one of the three β -subunits and the γ -subunit. Such a linkage stops the F_1 -ATPase in its tracks, presumably by welding the drive shaft to the engine block. After forming the linkage, complexes were chemically dissociated, then reconstituted, but this time the two unlinked β s were substituted with radioactively labelled subunits. The disulphide crosslinks were reduced, unfettering the enzyme, which was then supplied with ATP. After many rounds of catalysis, the crosslink was re-established by oxidation, and dissociation of the complex revealed an admixture of linked β - γ subunits — some radioactive and others not — that was quantitatively consistent with a random redistribution of the linkage sites among all three β -subunits. Although this did not prove that an ordered, sequential rotation occurs, it confirmed that the γ -subunit could visit each β -subunit freely, consistent with rotational catalysis.

Sabbert *et al.*¹² took advantage of another cysteine residue on the γ -subunit drive shaft to link up a maleimide-coupled eosin dye. By exciting the dye with a flash of polarized laser light from one direction, then recording the anisotropy change for the subsequent absorption of continuous polarized light coming from an orthogonal direction, they obtained signals that decayed over time and were consistent with rotation of the bound eosin chromophore through at least 200°. These signals depended on ATP, and the time constant for the decay was roughly

compatible with the known rates of ATP hydrolysis.

However, nothing is quite so compelling as seeing rotation with one's own eyes, and this is exactly what Noji *et al.*¹ have done. Once again, the crystal structure served to identify likely sites of attack. First, histidine tags were genetically engineered into the β -subunits on their cytoplasmic faces, so that F_1 complexes could be attached stereospecifically to a Ni^{2+} -coated surface, with their F_0 -binding faces pointing up, away from the surface. Second, a cysteine was engineered at position 107 of the γ -subunit and used to couple a short, fluorescently labelled actin filament (about a micrometre in length) to the drive shaft. Third, isolated complexes were bound at low surface-densities inside microscope flow-cells coated with Ni^{2+} , and viewed in fluorescence mode. In the presence of ATP, some of the actin filaments were driven round and round like microscopic propellers, powered by hydrolysis in the F_1 complex. The handedness of this rotation could be predicted in advance because (viewed from above) the catalytic sites are arrayed clockwise in the order: ATP-binding, ADP-binding and empty. So the reverse rotation induced when the F_1 -ATPase splits ATP (rather than when the H^+ -ATP synthase makes it) should carry the drive shaft around anticlockwise — and all of the spinning actin filaments indeed turned anticlockwise (Fig. 1, page 300).

Curiously, the angular speeds of filament rotation were rather constant, showing little tendency to slow down or speed up three times per revolution, contrary to what might be expected for a motor with only three 'pistons'. This might be due to thermal motion, but it could also be a clue to the molecular mechanism: more data are needed on speeds, torques and fluctuations. The flagellar motor also turns remarkably smoothly, despite having just eight force generators^{3,13}. The reported angular speeds for the F_1 -ATPase were relatively low (< 4 Hz) — solution hydrolysis rates would predict speeds nearer to 20 Hz. Enormous mechanical loads imposed by viscous drag on the actin filaments doubtless slowed things down. Noji *et al.*¹ estimated this drag to be over 90 pN nm Hz⁻¹ for a 2.6- μ m-long filament (Fig. 2, page 300), and the (unknown) proximity of filaments to the nearby surface may raise the value threefold; perhaps more.

A filament rotating at 0.5 Hz, therefore, dissipates an energy in excess of $(2\pi) \times (90 \text{ pN nm Hz}^{-1}) \times (0.5 \text{ Hz}) = 280 \text{ pN nm}$ per revolution. This should be compared with the chemical free energy available from hydrolysis, which is roughly 80 pN nm per molecule of ATP. Assuming tight coupling of rotation to hydrolysis, and exactly three ATPs per revolution, the chemical-energy input would come to 240 pN nm. So either the F_1 -ATPase is operating at 100 per cent

thermodynamic efficiency or, more likely, some 'slippage' occurs, such that more than three ATPs are consumed per revolution.

These ground-breaking experiments pose further questions and pave the way for studies using the F_1 rotation assay. The achievement of Noji *et al.*¹ adds to a burgeoning array of motility assays that are revolutionizing the study of macromolecular machines, including myosin, kinesin, dynein, RNA polymerase and the bacterial motor^{3,14,15} (Fig. 1 of this article, page 217). In conjunction with an arsenal of newly developed instruments, such as laser-based optical traps, scanned-probe microscopes, electrorotation apparatus and advanced fluorescence techniques, such assays make possible biophysical studies at the level of individual macromolecules¹⁴⁻¹⁸. □

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illustrated in Fig. 1. The figure shows a collection of atoms, pulled apart by some force, and with a crack reaching part way through. In a brittle material, the applied force makes the crack move ahead. In a ductile material, by contrast, rows of atoms slide past one another, and so the material responds to the applied force by making the crack blunter rather than longer^{4,5}. The sliding motion of the atoms is called dislocation motion, and the region along the plane of sliding where atomic bonds break and re-form is the dislocation.

Carrying out a calculation with any semblance of realism is much more involved than drawing a schematic picture. Zhou, Beazley, Lomdahl and Holian provide the largest-scale attempt yet. The setting for their calculation is a model block of copper 10^{-7} metres thick, and containing 35 million atoms. They arrange the atoms so that the copper has a crack in it, instruct the computer to pull on the top and bottom of the block, and follow the consequences for 25×10^{-12} seconds. Although considerably easier than modelling a whole penny, this calculation must overcome numerous difficulties.

The first challenge is to circumvent the impossible demands of quantum mechanics. Employing quantum mechanics slavishly, following only 35 million of the electrons in the copper and allowing them to explore only 10 states per atom would need $(350 \times 10^6)! / (35 \times 10^6)!(315 \times 10^6)!$ variables. With computers doubling in size every year, it will be more than 10^7 years before such a calculation comes within reach. Instead, the authors use the embedded-atom method⁶, which

Materials science

Breaking in computers

Michael Marder

Shujia Zhou, David Beazley, Peter Lomdahl and Brad Holian of Los Alamos National Laboratory have made a remarkable discovery¹. They have found that if you drop a penny on the floor, it won't snap in two. However, they didn't settle for the easy way, by emptying their pockets; they did it the hard way, on the computer.

There is no better example of the enormous scientific difficulties in demonstrating the obvious. Most solid objects fall naturally into the classes of brittle or ductile^{2,3}, but unlike many other qualitative material properties, such as colour and electrical conductivity, brittleness cannot easily be deduced from known physics at the atomic level. Colour and conductivity are already evident when a solid is subjected to very weak external forces — as applied by light or electrical current — so the adjustments to all the atoms are tiny, and

approximations work well. Brittleness and ductility only become evident in the presence of forces large enough to rip atoms apart, a much harder case to handle.

The most productive approach is to ask what happens to material with a crack, as

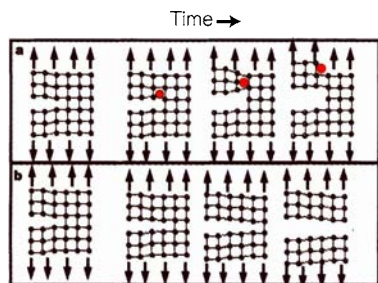


Figure 1 a, A crack in a ductile material emits a dislocation and blunts in response to stresses. The red dot marks a dislocation core. b, By contrast, cracks in brittle materials propagate freely.

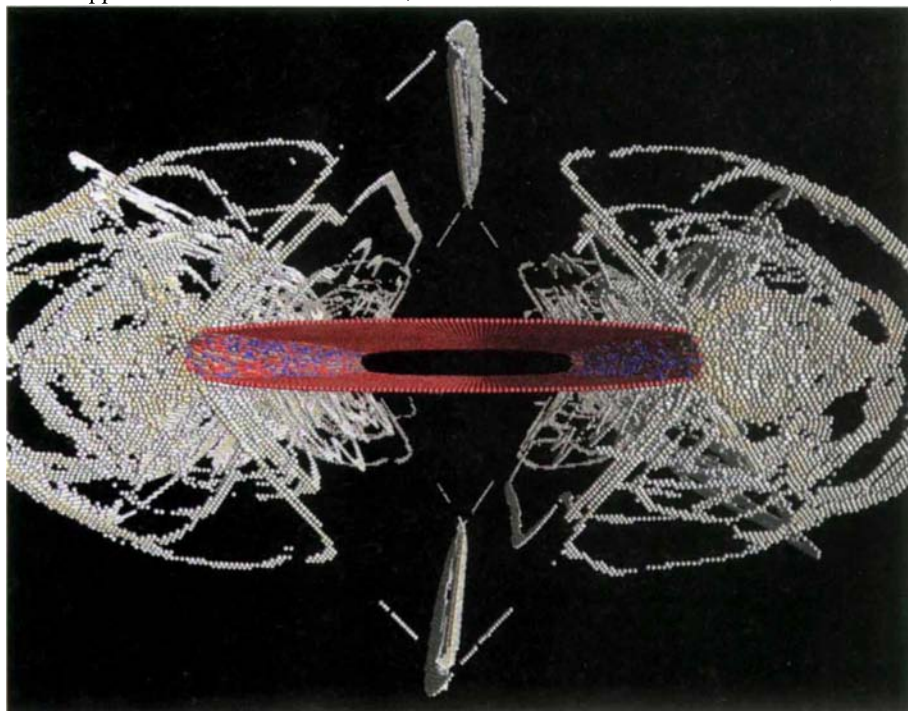


Figure 2 A three-dimensional simulation of a crack in a thin film of copper¹. Viewed along its axis normal to the film, the crack attempts to propagate horizontally; tension has been applied in the vertical direction. Only atoms in the film having higher potential energy than the bulk are displayed, including crack-surface atoms and dislocation cores, coloured according to their potential energies.

partially mimics quantum-mechanical complexity while nevertheless using no more than a few variables per particle. The simulated atoms move about in a pre-quantum fashion, obeying Newton's laws, but the forces they impose on one another incorporate information from quantum mechanics⁷.

The next difficulty is to write a computer program able to deal efficiently with tens of millions of atoms. Designed for use on the most recent classes of parallel machines, the code is named SPaSM (from "scalable parallel short-range molecular dynamics"). The problem in programming for parallel computers is that although ideally many different processors operate simultaneously, it can too easily happen that most processors are forced to wait idle while one or two stragglers finish essential tasks. But SPaSM can occupy all of the processors over 90% of the time⁸.

The final difficulty is to analyse and understand the results; if one wanted just to print out the locations of the particles at one time, and could fit 1,000 sets of coordinates on a page, the printout would be 35,000 pages long. It is crucial to have the computer itself reduce the information contained in the atoms' positions to a relatively small number of meaningful quantities. This final hurdle is cleared by having the computer prepare pictures that strip away almost all of the atoms, and draw only those right along the crack surface or tracing out the cores of the dislocations (Fig. 2, preceding page).

This figure leaves no doubt about the ductility of copper. Within the brief time interval of the simulation, a great tangle of

dislocations has poured out from the crack tip. Some resemble the one in the sketch of Fig. 1 in that they cause the crack to become progressively more blunt. Others emerge from the crack tip in a perpendicular direction, causing the crack line to bend and jog. The first dislocations to emerge from the crack travel upwards but, surprisingly, backwards, opposite to the expected direction of crack motion. By the end of the simulation, dislocations are bumping against the top of the sample; even more atoms would be needed to go further.

Such a computation is just the beginning of an endeavour to understand the subtle consequences of approximations; the beginning of comparison with experiment that may eventually lead to trustworthy predictions starting at the atomic level. Computers will never become large enough to preclude the need for thought and understanding, but the detailed information now being provided by Zhou, Beazley, Lomdahl and Holian could never be obtained in any other way. □

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Crystal engineering

Cooperative bonding affords a holesome story

Michael J. Zaworotko

Crystal engineering¹ offers a stimulating scientific challenge² and the intriguing but unsubstantiated promise of creating entirely new classes of functional solid. Rational strategies for designing solids would affect areas as diverse and commercially relevant as catalysis, materials science, chemical separation and drug selection³. In the context of separation, the terms 'open framework' and 'molecular sieve' have become synonymous with porous inorganic networks, in particular the class of aluminosilicate commonly called zeolites. But these notions could be set for a dramatic transformation with a discovery from the laboratory of James D. Wuest and co-workers⁴. Publishing in this week's *Journal of the American Chemical Society*, they describe a remarkably robust hydrogen-bonded porous crystal.

It is already clear that metal-organic

polymer frameworks can sustain channels^{5–7} or cavities⁸ that are as large as or larger than those found in naturally occurring zeolites.

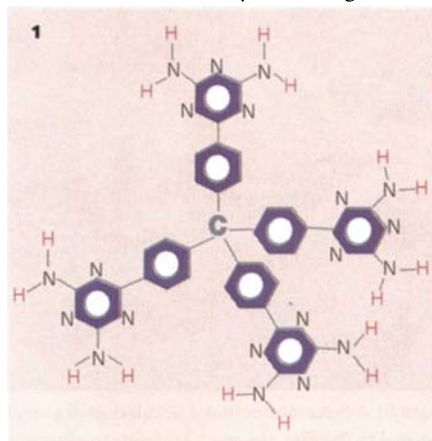


Figure 1 The many H-bonding sites of this molecule allow it to form robust porous crystals.

Furthermore, in at least two cases these new porous solids remain intact even after the partial loss of guest molecules such as benzene and pyridine^{5,6}. Wuest and his co-workers have synthesized an unlikely new member of this exclusive but growing class of compounds, an organic molecule that self-assembles to form a porous, three-dimensional, hydrogen-bonded network. The molecule (1; Fig. 1) is pseudo-tetrahedral, with many complementary hydrogen-bond donors (white) and acceptors (blue) on its periphery. It should therefore be no surprise that it self-assembles to afford a hydrogen-bonded network. But the network has some unusual properties.

The molecule crystallizes into a hydrogen-bonded framework of sheets that contain roughly square cavities, about 11.8×11.8 Å. These sheets crosslink, again by multiple hydrogen bonds, so that the cavities eclipse one another. The result is that the cavity becomes an infinite microchannel (Fig. 2). The inner walls of the channel contain the excess hydrogen-bond-donor sites present on 1, so they efficiently bind solvent molecules such as 1,4-dioxane, acetonitrile and water. The crystals lose solvent under vacuum, but retain their crystalline form even after losing 66% of the dioxane molecules.

The last is a most remarkable feature, because there had been no precedent for hydrogen bonds strong enough to sustain such a large amount of open space in a crystal. The key to the robustness of the network formed by 1 appears in hindsight to be quite simple: multiple hydrogen-bonding sites can provide enough mechanical strength to withstand the loss of a considerable portion of the contents of the crystal lattice. Similar stability is observed in the (covalently bonded) zeolites, and it is critical in catalysis and filtering because it makes adsorption and desorption of guest molecules a reversible process. Multiple

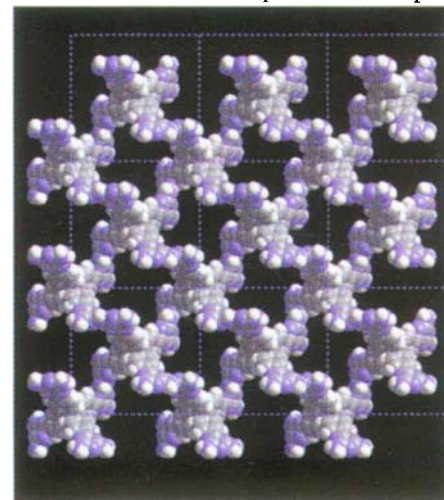


Figure 2 A view down the channels formed in crystals of molecule 1. Guest molecules are omitted for clarity.

hydrogen-bonding sites are also used for structural control and molecular recognition in important biological molecules such as DNA and proteins.

Whither crystal engineering? Crystal engineers are not yet able to rationally design molecular sieves for organic molecules of industrial and environmental relevance. But this new work greatly expands the playing field. Specifically, it is now clear that crystal engineers are not limited by the chemical nature of a species or the strength of the individual bonds that can be exploited to sustain porous networks. So it is not hard to be optimistic about the prospects for crystal engineering, especially when one considers that many of the computational and experimental tools required are available as never before.

Finding new classes of molecular sieve that are inherently designed and fine-tuned for medium to large organic molecules (such

as polychlorinated biphenyls or polycyclic aromatic hydrocarbons) is just one obvious short-term goal for the crystal-engineering community. When one considers the chemical diversity and unnatural architecture of many of the networks now being, dare we say it, designed, the long-term future of crystal engineering seems likely to be full of unexpected developments. □

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Excitatory synapses

Glutamate receptors put in their place

Morgan Sheng

Nerve cells transmit signals to one another through synapses. Effective transmission of these impulses requires the precise localization and high concentration of neurotransmitter receptors on the receiving, postsynaptic, side of the synapse. The molecular mechanisms of this targeted clustering have been long sought after, and two papers in this issue — those by Dong *et al.*¹ (page 279) and Brakeman *et al.*² (page 284) — advance our understanding of the process. The two groups have identified hitherto unknown proteins that bind specifically to two types of glutamate receptor. In doing so, these studies highlight the importance of a modular protein-interaction domain (the PDZ domain) in the molecular organization of synaptic junctions (Fig. 1).

Glutamate, the major excitatory neurotransmitter in mammalian brain, binds to and activates several classes of postsynaptic receptor. Two types (NMDA receptors and AMPA receptors) are ligand-gated ion channels that transduce glutamate binding into cation influx into the postsynaptic cell. A third class, the metabotropic glutamate receptors, belong to the G-protein-coupled seven transmembrane receptor superfamily; these mediate the slower actions of glutamate through intracellular second messengers. (Inhibitory synapses use different neurotransmitters and different receptors. The latest development, cloning of a receptor subtype involved in inhibitory synapses, is pub-

lished elsewhere in this issue — see pages 239 and 223.)

At glutamate excitatory synapses, neurotransmission clearly depends on the different kinds of receptor being concentrated at appropriate postsynaptic sites where they can respond to synaptically released glutamate. Such postsynaptic clustering has been inferred from electrophysiological studies

and shown directly by immunocytochemistry.

How does this specific localization occur? The first step forward came with the discovery that NMDA receptors bind to the PSD-95/SAP90 family of synaptic proteins^{3,4}. The PSD-95 protein contains three PDZ domains that specifically bind to peptide sequences at the very end of the carboxy terminus of interacting proteins. For instance, the carboxy terminus of NMDA receptor NR2 subunits (amino-acid sequence -ESDV, in single-letter code) interacts with the first two PDZ domains (PDZ1 and PDZ2) of PSD-95. The same two PDZ domains also allow PSD-95 to bind to Shaker-type K⁺ channels, which end in the sequence -ETDV (ref. 5). PSD-95 can organize clusters of NMDA receptors and Shaker K⁺ channels in heterologous cells⁶. More significantly, *Drosophila* mutants of the fly homologue of PSD-95 show loss of synaptic clustering of Shaker channels⁷. By extrapolation, one supposes that PSD-95 is important in the clustering of NMDA receptors in mammalian synapses.

So much for NMDA receptors — what about the other kinds of glutamate receptor? Although AMPA receptors coexist with NMDA receptors in glutamatergic synapses, they do not bind PSD-95. AMPA receptor subunits do not have carboxy-terminal sequences that 'fit' the PDZ domains of PSD-95. Instead, Dong *et al.*¹ have discovered that AMPA receptor subunits (with the carboxy-terminal sequence -SVKI) bind to a novel synaptic protein called GRIP (for glutamate receptor interacting protein). GRIP contains seven PDZ domains, making it the numerical leader in the PDZ stakes,

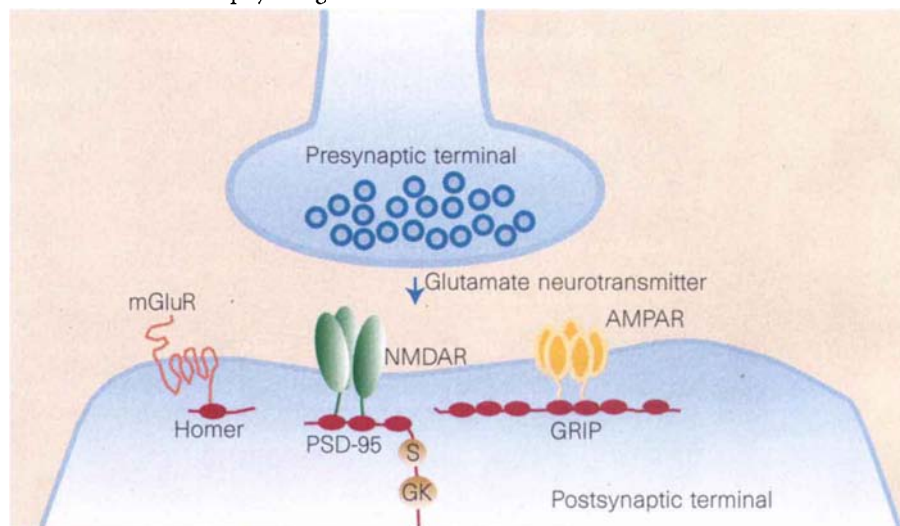


Figure 1 Clustering of glutamate neurotransmitter receptors on the postsynaptic side of an excitatory synapse, as mediated by PDZ (or functionally PDZ-like) protein-interaction domains. The metabotropic glutamate receptors (mGluR), NMDA receptors (NMDAR) and AMPA receptors (AMPA) bind to specific PDZ domains of the proteins Homer, PSD-95 and GRIP, respectively. The remaining PDZ domains of PSD-95 and GRIP presumably bind to other synaptic proteins. The receptors differ in their subsynaptic distribution, mGluRs being located at the periphery, whereas NMDARs and AMPARs are more central (ref. 10). The PDZ domains are shown in red. S, SH3 domain; GK, guanylate kinase domain.

but is otherwise unrelated to PSD-95. Only the fourth and fifth PDZ domains of GRIP are involved in binding to the carboxy-terminal tail of AMPA receptor subunits; the remaining PDZ domains are presumably responsible for interacting with other unidentified proteins. Thus, GRIP is likely to be an adaptor protein that physically and functionally couples AMPA receptors to a variety of cytoskeletal and signalling molecules. Rounding up all these GRIP-interacting proteins is certain to improve our appreciation of the architecture of glutamatergic synapses.

In cultured neurons, Dong *et al.*¹ find that GRIP colocalizes with AMPA receptors at synaptic sites, and that overexpression of the carboxy-terminal tail of the AMPA receptor subunit GluR2 causes loss of synaptic AMPA receptor clusters. Formally, this nice experiment confirms the importance of the GluR2 carboxy-terminal tail, rather than of GRIP, in AMPA receptor targeting. Nevertheless, even though the evidence is not watertight that GRIP is essential for the synaptic clustering of AMPA receptors, the idea is made compelling by analogy to the PSD-95 story.

Brakeman and colleagues² further extend the theme of glutamate receptor clustering by PDZ-containing proteins — this group has isolated a protein, called Homer, that binds to a subset of metabotropic glutamate receptors (mGluR1 α and mGluR5). Binding to Homer requires the carboxy terminus of mGluR1, the sequence of which (-SSSL) looks suspiciously like a PDZ-binding motif⁶. Unlike PSD-95 and GRIP, Homer is a small protein (relative molecular mass 28K) and contains a single 'PDZ-like' domain. The reason for the reservation is that Homer's PDZ domain contains little sequence similarity to known PDZ domains, except for the presence of a GLGF sequence and an arginine a few residues upstream. In the crystal structure of a PDZ domain from PSD-95, these conserved residues form a loop that interacts with the carboxy-terminal carboxylate group of the bound peptide⁹.

Whether Homer possesses a highly divergent PDZ domain, or a new type of carboxy-terminal peptide-binding domain, remains to be seen. Regardless of the answer, a fascinating feature of Homer is that its expression level is dramatically induced by synaptic activity. Assuming that Homer is involved in synaptic localization of mGluRs, the direct implication of this finding is that neuronal activity can regulate the synaptic clustering of certain metabotropic glutamate receptors. Activity-dependent redistribution of channels and receptors in synapses has been invoked to explain some aspects of synaptic plasticity — Homer looks like a potential mediator of such structural changes.

A final question. Why are three different proteins involved in the postsynaptic clustering of NMDA-, AMPA- and metabotropic-type glutamate receptors? One reason for such variety is that it allows differential distribution of these receptors within the same glutamatergic synapse, for which there is direct electron microscopic evidence¹⁰. Another is to allow differential regulation of receptor density in the postsynaptic membrane. A third is that GRIP, Homer and PSD-95 may function as adaptor proteins that physically couple the different classes of glutamate receptors to distinct downstream signalling pathways. Clearly, the identification of GRIP and Homer is just a sneak preview of the molecular complexity of neuronal synapses yet to come. □

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Inhibitory synapses

The cloning of GABA_B receptors

Norman G. Bowery and David A. Brown

GABA (γ -aminobutyric acid) is the brain's main inhibitory neurotransmitter. It acts on two different types of target receptor: GABA_A and GABA_B. The GABA_A receptor was cloned a decade ago, but the GABA_B receptor has defiantly resisted the cloner's embrace — that is, until now. On page 239 of this issue, Kaupmann *et al.*¹ report that they have cloned two forms of rat GABA_B receptor complementary DNA using a new high-affinity antagonist for the GABA_B receptor.

The GABA_A receptor belongs to the broad class of multi-subunit proteins that form 'ligand-gated' ion channels² (Fig. 1a, overleaf). When the receptor is 'gated' (opened) by GABA, chloride ions flow through the channel, usually into the cell. This hyperpolarizes the cell membrane and produces a rapid cessation of the cell's electrical discharge.

The GABA_B receptor (Fig. 1b) works quite differently — it links to another membrane protein (a guanine-nucleotide-binding (G) protein) to produce several divergent effects: opening of adjacent potassium channels; closure of voltage-gated calcium channels; and inhibition (or sometimes activation) of the enzyme adenylyl cyclase³. As a result, the effects of stimulating GABA_B receptors are slower (because it takes time to complete the connecting steps) and more diverse than those that follow the stimulation of GABA_A receptors. For example, activation of GABA_B receptors on a recipient cell membrane produces a slower, more prolonged postsynaptic inhibition. There are also presynaptic GABA_B receptors on the terminals of axons which release GABA (or other transmitters). When these are stimulated, the release of transmitter is reduced,

possibly because of the effects on calcium and/or potassium channels, or through other means⁴.

A presynaptic G-protein-coupled GABA_B receptor that was selectively stimulated by the GABA analogue baclofen (β -*p*-chlorophenyl-GABA) was identified in the early 1980s^{5–7}. Since then, a plethora of such G-protein-linked receptors has been cloned, including receptors for GABA's excitatory counterpart, glutamate^{8,9}. So was the GABA_B receptor to be the last wallflower at the receptors' dance?

The answer is no. Kaupmann *et al.*¹ have cloned rat GABA_B receptor cDNAs by expression cloning in mammalian COS-1 cells, using the high-affinity antagonist [¹²⁵I]CGP64213. Starting with a cDNA library from rat cerebral cortex and cerebellum, they first isolated a single clone containing a 4.4-kilobase cDNA encoding a 960-amino-acid (relative molecular mass, 106K) protein. Further screening of the cDNA library by hybridization with this probe revealed a shorter form (2.9-kb cDNA encoding an 844-amino-acid protein, *M*_r 92K). These corresponded to the deglycosylated relative molecular masses of two proteins that the authors isolated from brain tissue by photoaffinity labelling.

The two forms, designated GABA_BR1a and GABA_BR1b respectively, differ only in the length of their amino-terminal sequences. They are pharmacologically identical, and show similar ligand-binding affinities. Binding affinities for a range of antagonists also match those reported for native GABA_B receptors, although the agonist-displacement constants are lower than those observed with native receptors — perhaps because of limited G-protein-coupling

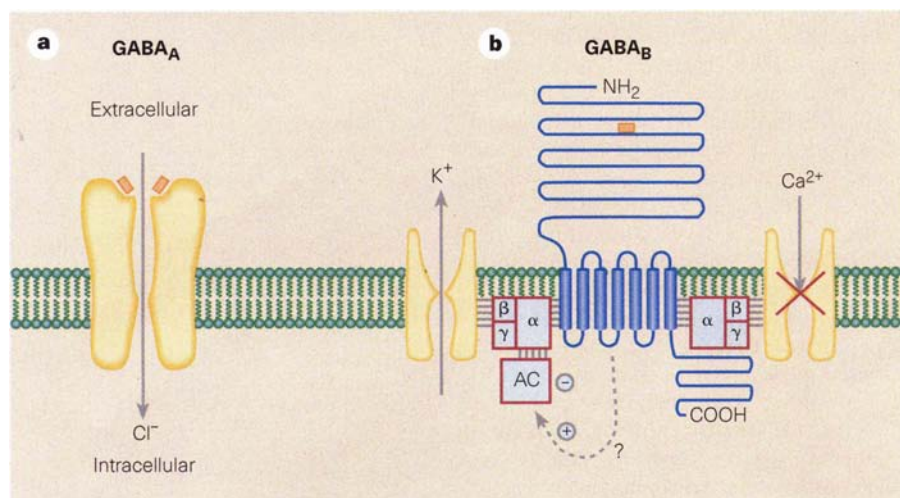


Figure 1 GABA (γ -aminobutyric acid) acts on two types of target receptor. **a**, The GABA_A receptor. Two molecules of GABA (squares) bind to the α -subunits of the pentameric ion-channel receptor and open the channel to allow an influx of chloride ions. **b**, The GABA_B receptor, which has now been cloned by Kaupmann *et al.*¹. One molecule of GABA binds to the large extracellular domain of the monomeric GABA_B receptor, which has seven transmembrane helices. The activated receptor then couples to the α -subunit of associated G proteins. One of these (G_i) inhibits adenylyl cyclase (AC), probably through its α -subunit, and opens a potassium channel (probably through its $\beta\gamma$ subunits)¹⁵. Another G protein (G_o)¹⁶ retards the opening of a calcium channel, probably through its $\beta\gamma$ subunit^{17,18}. Stimulation of adenylyl cyclase has also been reported, through an undefined connection. (A third class of GABA receptor — the GABA_C receptor — has also been advocated¹⁹. However, despite their unique pharmacology and subunit make-up, GABA_C receptors structurally resemble GABA_A receptors. As Kaupmann *et al.* have now revealed the major structural difference between the GABA_A and GABA_B receptors, GABA_C receptors should be designated as a subgroup of the GABA_A -receptor class.)

in the cell lines used for expression. (A little pharmacological wrinkle emerges here: displacement curves for the frequently used 'antagonists' saclofen and 2-hydroxy-saclofen line up better with the agonists than with the other antagonists, suggesting that these may be partial agonists, rather than true antagonists.)

Do the receptors work as they should? It seems that they do — at least, partly. In human embryonic kidney cells, they inhibited adenylyl cyclase through the expected pertussis-toxin-sensitive G protein, although there was no evidence for activation of adenylyl cyclase. Moreover, the authors could not show functional coupling with membrane channels in *Xenopus* oocytes. But many groups have been unable to obtain reliable functional expression of GABA_B receptors in these amphibian cells using crude mRNA from rat cerebral cortex or cerebellum, even when the expected target G proteins and potassium channels were coexpressed. The reason for this is unclear, but this refractoriness of the oocyte expression system undoubtedly contributed to the long pre-cloning gestation period.

Kaupmann *et al.*¹ point out that the GABA_B receptor proteins are bigger than most other G-protein coupled receptors, with the exception of the metabotropic receptors for glutamate (mGluRs)^{8,9} (the functioning of glutamate receptors is discussed on pages 279, 284 and 221 of this

issue). Not only are the GABA_B receptors of a similar size to mGluRs, but they have a similar 'structural architecture' and, in both cases, there is homology between the amino-terminal extracellular domain and bacterial amino-acid-transport proteins (suggesting that the amino terminus is the site for GABA binding). On reflection, these similarities are not so surprising, as the glutamate and GABA transmitter systems have a long evolutionary history and could be described as 'old' receptors to contrast with the 'younger' neuropeptide systems. There are, nevertheless, marked differences in the sequences; for example, in the second intracellular loop, which determines the G-protein coupling in mGluRs.

Where are the GABA_B receptors? Pretty much where they should be, judging from the beautiful $\text{GABA}_B\text{R1}$ mRNA hybridization pictures from Kaupmann *et al.*, which complement previous receptor autoradiographic studies. Notably, GABA_B mRNA is detected in Purkinje cells and the granular layer of the cerebellum, and the medial habenular, in exact agreement with the receptor-protein-binding data^{10,11}.

The new sequence data should (in time) help to answer various questions. How many GABA_B receptor subtypes are there? Where are they located? Are they coupled to different effector mechanisms, or does the same GABA_B receptor couple to potassium and calcium channels? Are the GABA_B receptors

found on presynaptic terminals the same as those at postsynaptic sites? And are all of the presynaptic receptors the same, or do GABA_B receptors on terminals that release GABA itself (autoreceptors) differ from those on terminals that release other transmitters (heteroreceptors)? It has already been suggested that GABA_B receptors controlling different release processes in rat brain synaptosomes can be subclassified into at least four types¹² and, in brain slices, the inhibition and stimulation of cyclic-AMP formation may be mediated through distinct GABA_B receptor subtypes¹³.

In animal models, antagonists to the GABA_B receptor can block absence-epilepsy seizures and increase cognitive performance. But the currently available antagonists are not specific for presynaptic (hetero- and autoreceptors) and postsynaptic GABA_B receptors. For example, in the hippocampus, the generation of long-term potentiation can be readily modulated by GABA_B antagonists¹⁴: perhaps a selective GABA_B antagonist acting at pre- rather than postsynaptic sites would augment long-term potentiation and so provide a lead in the search for new cognitive enhancers.

This selectivity concept may equally apply to receptor agonists. Although baclofen — the archetypal agonist — is used therapeutically, it does have unwanted side effects in some people: these might be minimized by focusing on a single GABA_B receptor subtype. However, as receptor subtyping is the nutrient that feeds the modern pharmaceutical industry, by providing the tool to pull out new subtypes (or to resolve old ones), the new sequence from Kaupmann *et al.*¹ offers exciting prospects for future drug targeting. □

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Time to trap an ytterbium ion

Pauline Rigby

A single ion held motionless in an electrodynamic trap may provide the ultimate standard for measuring time¹. The current standard is defined in terms of the energy separation between two hyperfine energy levels in the ground state of a caesium atom (Fig. 1). At the moment, the best caesium clocks^{2,3} are accurate to within three parts in 10^{15} , but the detection of a new atomic transition at the UK National Physical Laboratory, reported in *Physical Review Letters*⁴ this month, sets the scene for an optical clock more than 1,000 times as accurate. Such high accuracy is equivalent to an error of less than one second in measuring the age of the Universe.

When an electron moves between two energy levels of an ion, it absorbs or ejects a photon whose energy matches the difference between the two levels. To be a suitable candidate for an optical frequency standard, the ion must possess a long-lived or metastable level, and the photon wavelength produced by the transition between this metastable level and the ground state must be accessible to continuous-wave lasers. The rare-earth metal ytterbium contains three potentially suitable transitions: at 411, 435 and 467 nanometres. The first two of these have already been observed^{5,6}, but the third has eluded detection until now. But it is precisely because it is so difficult to observe that the transition is so interesting.

Roberts *et al.*⁴ have seen the transition for the first time, and found that the upper state lives for about ten years. This is the time it takes for an electron to fall spontaneously back to the ground state, a phenomenon called fluorescence. If we know the lifetime

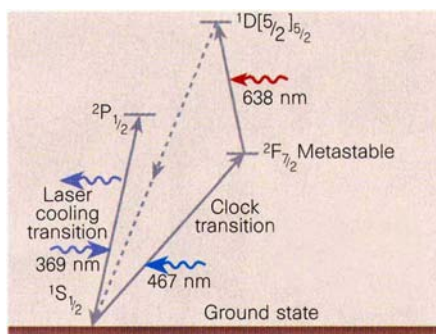


Figure 2 Simplified representation of energy levels in an ytterbium ion. An electron is cooled with ultraviolet light, driven into the metastable level with blue light and finally recycled with red light.

of the state, then its linewidth — the spread of energies about a central frequency — is fixed by Heisenberg's uncertainty principle. The more familiar form of the principle says that the more accurately the position of a particle is determined, the more uncertain we must be about its momentum. Another form of this law relates time and energy. The product of the two variables, position and momentum or lifetime and linewidth, has a fixed value. Because the lifetime of the 467 nm transition is so long, it is extremely narrow in frequency — perhaps the narrowest spectral line available.

The key parameter for the stability of a clock is its *Q* factor, which is the ratio of the transition frequency to the linewidth, or the proportional uncertainty in the frequency of a single emitted photon. For an ion under normal atmospheric conditions there are many mechanisms that will broaden the linewidth, such as Doppler (motion induced) broadening. But the Doppler effect can be exploited to eliminate itself and bring the ion to rest inside a trap, by the process of laser cooling. A laser with a wavelength slightly off the strong 369 nm transition is used. Only when the ion is moving into the laser beam (as it vibrates) does the laser frequency appear to match the transition and a photon can be absorbed. When the ion fluoresces, on average it releases a slightly more energetic photon than the one it received, so that over time the ion's vibrational energy is gradually depleted.

Once the ion is cooled down to a few millikelvin and trapped in a space whose dimensions are smaller than the wavelength of the laser light, then the frequency of the clock transition can be located. The region around the transition is scanned in small steps with a second laser beam which has a similarly narrow linewidth (otherwise it is like trying to pick up a pin with boxing gloves on). To scan a wide region about the clock transition with

enough care to be sure they hadn't missed it would have taken several years, so the group first narrowed down the search region, from 2.6 GHz to 8 MHz, by measuring two other connected transitions and subtracting the frequencies^{5,7}.

Fluorescence from the laser cooling transition is used to indicate when a 467 nm photon has been absorbed. A 369 nm laser and the scanning probe laser illuminate the sample alternately. The electron continually cycles up and down the 369 nm transition, fluorescing each time it falls back to the ground state, until the 467 nm transition gets resonantly excited by the probe laser (Fig. 2). Then the fluorescence stops because the electron gets stuck in the long-lived state.

Now the problem is how to return the ion to its ground state so that the scan can continue. One option would be to wait ten years, but this is obviously impractical. A third laser beam is used to send the electron on — up to a higher energy state with a short lifetime. It quickly falls back to the ground state from there, and the process can begin again.

Initially, the number of transitions observed was only one per hour, but this has now been increased to two per minute which has made it possible to build up a spectral profile of the clock transition and measure its frequency to better than 1 MHz, not far off one part in a billion. This is the first time that such a weak, so-called 'octupole' transition has been driven.

There is still a lot of work to be done before they can make a clock. The rate of jumps must be increased, by using a laser with an even narrower linewidth to locate the clock transition more precisely. The National Physical Laboratory is also working on more accurate methods for down-converting trapped ion optical frequencies to microwave frequencies for comparison with the caesium standard there.

Why do we need better clocks? No doubt physicists will rub their hands with glee at the prospect of devising new experiments to test the theory of relativity. Cold, trapped ions also present the ideal medium for finding out whether the fundamental physical constants, such as Planck's constant, really are constant. Satellite-based global positioning systems and long-baseline interferometry in radio telescopes are among the many applications that will benefit from the advance in accuracy of measuring something as fundamental as time. □

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IMAGE
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Figure 1 Old timer. This is the first caesium atomic clock, developed in 1955. A new standard may be set by single-ion ytterbium resonators.

An ice age in the tropics

Alan J. Kaufman

One of the enduring enigmas of Proterozoic Earth history (up until almost half a billion years ago) is the intimate association of glacial deposits with carbonates that appear to have formed in warm tropical seas. This palaeoclimatic paradox implies that, billions of years ago, Earth's surface thermostat was occasionally turned down low enough to allow glaciers to spread from the poles to very near the Equator, blanketing most of the planet in ice and snow. In contrast, the glaciers that scoured the Northern Hemisphere over the past few million years only reached as far south as the Ohio River valley in North America, and a line from near London to Kiev in Europe. The paradox might instead be explained by the formation of ancient carbonates in cold water at high latitudes, but support for at least one "Snowball Earth"¹ comes from a new palaeomagnetic study by Evans *et al.* on page 262 of this issue² — it suggests that glaciers covered most of southern Africa over two billion years ago, when that region sat roughly 11 degrees from the Equator.

Surface temperatures must have dropped considerably for glaciers to have existed in the tropics. The cause of the temperature drop was probably a decline in the atmospheric level of carbon dioxide, the greenhouse gas believed to be the main regulator of Earth's climate. The transfer of water from the oceans to a global network of Proterozoic glaciers would have lowered relative sea level, making the ocean more dense and salty; and the presence of thick, extensive sea ice might have caused the oceans to stagnate, by slowing down wind-driven circulation and blocking the sunlight that fuels biological photosynthesis and oxygen production. Covered with ice, the Earth would have reflected most of the Sun's warming rays back into space. Only a catastrophic event, like a huge volcanic eruption, a comet or asteroid impact, the sudden release of methane hydrates, or the overturn of a deep, stagnant ocean, could have pumped enough carbon dioxide back into the atmosphere to melt the planet's icy shell.

Some time after the Palaeoproterozoic ice age in Africa, molten rock erupted onto the surface to form the widespread Ongeluk Formation. Evans and colleagues make a strong case that this thick pile of volcanic rocks has remained largely unaltered, so that after two billion years certain iron-rich minerals still record the direction of Earth's magnetic field, like tiny compasses frozen in time. The angle of the preserved magnetic field is used to determine the latitude at which the rocks formed. The authors further suggest that there is no significant hiatus between these

tropical lavas and the underlying glacial rocks. That is critical, because the age of this glaciation is not well known. A 20- to 40-million-year break between the ice age and the volcanic episode would have allowed the southern African continent enough time to travel from high to low latitudes. If, on the other hand, there is no time missing between the two units, the thick lavas may mean that the ice age was ended by the volcanic release of massive amounts of carbon dioxide to the atmosphere.

There are several other Palaeoproterozoic glacial deposits recognized worldwide, although the exact correlation of the ice ages that they mark is not well established. In Ontario and Wyoming, for example, there is evidence of three discrete Palaeoproterozoic glaciations (Fig. 1), but which, if any, of these matches the South African example is difficult to say. Immediately overlying the second of the North American glacial deposits is a layer of the enigmatic carbonates

noted above, which are distinctive in both their fine-grained texture and chemistry, specifically in their carbon-isotope compositions^{3,4}. Relative to most carbonates deposited in the oceans over the past 2,500 million years, these 'cap' carbonates atop both Palaeoproterozoic and Neoproterozoic glacial rocks have consistently low $\delta^{13}\text{C}$ (a measure of the ratio of ^{13}C to ^{12}C ; see Fig. 2), suggesting that they were formed under very strange circumstances, by modern standards.

In the Neoproterozoic, where palaeomagnetic data also point to two low-latitude glaciations^{5,6}, strong positive-to-negative $\delta^{13}\text{C}$ excursions in carbonates bracket at least four discrete ice ages^{7,8}; and the highly positive $\delta^{13}\text{C}$ values beneath Neoproterozoic glaciations match those in some Palaeoproterozoic carbonates⁹. At present, however, poor age constraints and stratigraphic uncertainties make it unclear whether these older biogeochemical event markers precede or post-date glaciation.

The remarkable positive-to-negative trends in $\delta^{13}\text{C}$ encompassing Proterozoic glaciations suggest that carbon cycling is at the heart of the palaeoclimatic paradox. These variations are consistent with the

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Figure 1 The smooth surface of a nearly two-billion-year-old glacial deposit in Ontario, Canada, polished and striated by modern continental glaciers. (Photo courtesy of P. F. Hoffman.)

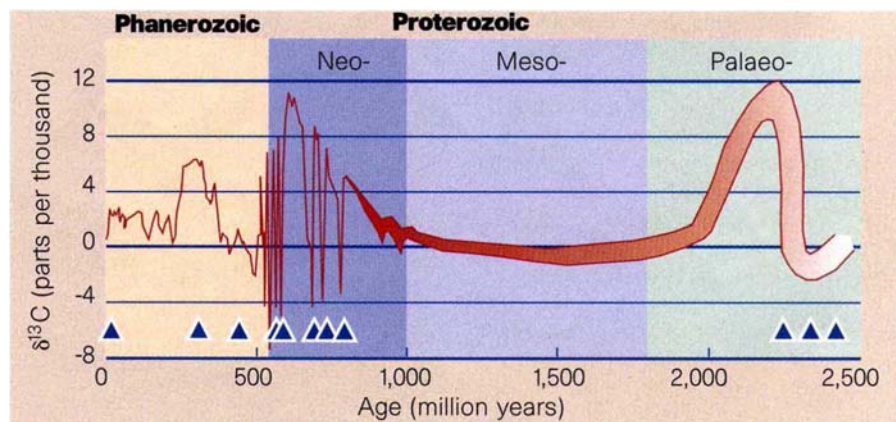


Figure 2 Carbon-isotope variation in sea water over 2,500 million years, as determined by the analysis of marine carbonates. Strong positive-to-negative trends in the relative isotope ratio $\delta^{13}\text{C}$ bracket Neoproterozoic (and perhaps Palaeoproterozoic) glacial deposits (see triangles), and suggest that carbon cycling is at the heart of long-term global climatic change.

hypothesis that high biological productivity (which effectively distils ^{12}C from sea water) in nutrient-rich Proterozoic oceans drove the $\delta^{13}\text{C}$ of surface water to positive extremes before the glaciation, helping the growth of continental ice sheets by removing carbon dioxide from the atmosphere^{7,8}. The negative $\delta^{13}\text{C}$ values in the cap carbonates that follow these ancient glaciations might best be explained by the rapid mixing of shallow seas with deep oceans that are chemically and isotopically distinct^{7,8,10,11}. Note that this process, which assumes that post-glacial Proterozoic oceans were somehow stirred more rapidly as the glaciers melted and surface temperatures warmed, is the opposite of how oceans are thought to have mixed after the most recent glaciations. If the ancient deep ocean was also a storehouse of carbonate alkalinity^{10,11}, degassing and the rapid precipitation of the cap carbonates during post-glacial mixing would have pumped enough carbon dioxide back into the atmosphere to ensure an end to the ice age.

The chilling possibility of Proterozoic glaciers in the tropics, inferred from palaeomagnetic data and from carbon-isotope evidence that is consistent with biological control of ice ages, highlights the many differences between recent and ancient Earth

history. In particular, models that attribute the drawdown of atmospheric carbon dioxide — and the subsequent growth of continental glaciers — to increased chemical weathering of young mountains (over the past few million years, the Himalayas and Tibetan Plateau¹², for example) may be inappropriate for our understanding of long-term global climate change. □

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Dosage compensation

Remodelling chromatin with RNA

Huntington F. Willard and Helen K. Salz

One of the fundamental differences between males and females is the number of X chromosomes. Because some functions of the X chromosome require equal expression in the two sexes, several types of dosage-compensation system have evolved that have the effect of attenuating the possible phenotypic consequences of this difference in chromosome content. In the fruit fly *Drosophila*, transcription of most of the genes on the single male X chromosome is upregulated compared with expression from each of the two female Xs (Fig. 1). In nematodes, by contrast, genes on the two hermaphrodite X chromosomes are downregulated compared with genes on the single male X. And in mammals, most of the genes on one of the two female X chromosomes are not transcribed at all due to X-chromosome inactivation (reviewed in ref. 1). Although these three approaches to dosage compensation — hypertranscription, hypotranscription and transcriptional repression — are not obviously related mechanistically, studies including the papers by Herzing *et al.*² and Lee and Jaenisch³ on pages 272 and 275 of this issue, and two reports in *Cell*^{4,5}, indicate a possible link: the complexes that are used to remodel chromatin structure on the X chromosome in each system show intriguing and

unexpected parallels involving non-coding RNA molecules.

The mechanisms of dosage compensation are each reflected in changes in the structure of chromatin in the X chromosome. In the interphase nucleus of a female mammalian cell, the inactive X chromosome takes on a distinct heterochromatic form, which was recognized as the Barr body as early as 1949 (ref. 6). In *Drosophila*, the 'bloated' appearance of the single male X chromosome compared with the two female X chromosomes was first noted in the 1930s, and it was attrib-

uted by Dobzhansky⁷ to the differential accumulation of 'some products of gene activity' in the two sexes. Studies from Meyer and colleagues indicated that dosage compensation in nematodes is accomplished by the differential condensation of chromatin on the X chromosome in that organism as well^{8,9}.

In mammals, X-chromosome inactivation is under the control of a single *cis*-acting locus called the X-inactivation centre. Within this region, the most likely candidate identified to date is the non-coding *Xist* gene, which is expressed only from the inactive X chromosome, and has been implicated in the establishment of X inactivation on the basis of genetic and developmental data from humans and mice¹⁰. The RNA product of *Xist* associates closely with the inactive X chromosome in an RNA-Barr body complex. Now, Herzing *et al.*² and Lee and Jaenisch³ have introduced ectopic copies of *Xist* into autosomes in murine embryonic stem cells, and they have recapitulated in these autosomes almost all of the molecular and chromosomal features that characterize X inactivation — namely, expression of *Xist* and localization of the *Xist* RNA in *cis*, gene inactivation, late DNA replication, and histone H4 hypoacetylation.

At least some of these effects can be seen with a cosmid containing the *Xist* gene alone², indicating that *Xist* probably is the X-inactivation centre and that it acts at the RNA level to remodel chromatin and to promulgate the X-inactivation signal along one of the female X chromosomes (Fig. 2). But significant questions remain. To what extent is the *Xist* RNA a required part of the chromatin-remodelling complex? Also, what other partners might be part of such a complex, and how does this suspected ribonucleoprotein-chromosome complex work in the context of the Barr body?

In flies, it is the genes on the male X chromosome that undergo chromosome-specific regulation. Four genes have been identified which are only required for hypertranscription of the male X chromosome, and they are named for their mutant phenotypes: *male-specific lethal-1*, *male-specific lethal-2*, *male-*

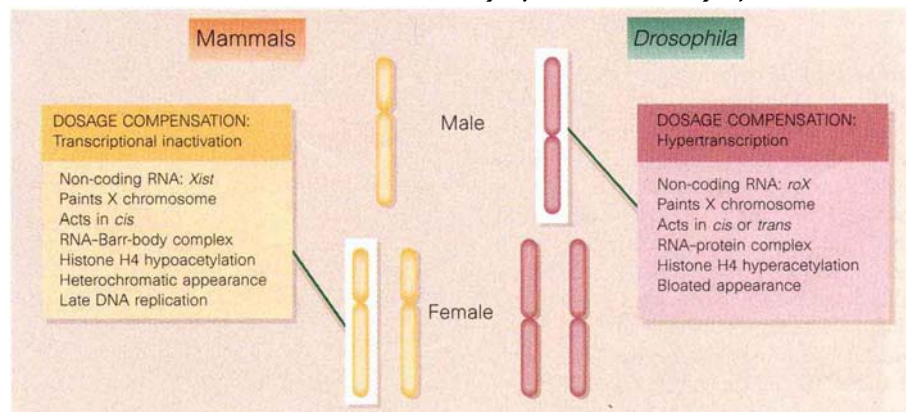


Figure 1 Comparison of dosage-compensation mechanisms and consequences in mammals and *Drosophila*.

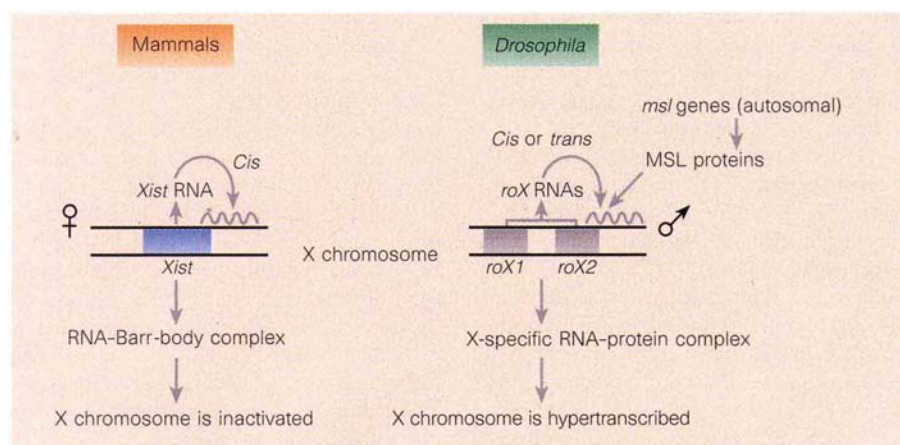


Figure 2 Herzing *et al.*² and Lee and Jaenisch³ have introduced ectopic copies of the *Xist* gene into murine stem cells to show that *Xist* regulates mammalian X-chromosome inactivation. Following transcription, the *Xist* RNA interacts with (or 'paints') the female X chromosome, thereby inactivating it. Meller *et al.*⁴ and Amrein and Axel⁵ report that dosage compensation in *Drosophila* occurs by a mechanism that also involves non-coding RNAs. The RNA products of the *roX* genes paint the male X chromosome in a similar manner to *Xist*, but this localization depends on the products of the *msl* genes and results in hypertranscription of the X chromosome.

less and maleless on the third. These autosomal genes — collectively known as the *msl* genes — encode *trans*-acting proteins that associate with (that is, 'paint') the X chromosome along its length. Sex-specific localization of an MSL protein is interdependent on the other proteins, so a mutation in any one of the *msl* genes will abolish the X-chromosome localization of all of the MSL proteins. Together with immunoprecipitation data, these observations indicate that the four MSL proteins form a complex that can bind to the male X chromosome, to alter the chromatin structure and allow increased transcription (Fig. 2). Moreover, histone H4 is hyperacetylated on the male X chromosome in an MSL-dependent manner, consistent with a direct effect of the four MSL proteins on remodelling the chromatin architecture (reviewed in refs 1 and 11).

Two further papers, in *Cell*, have built a case that, just as in mammalian X-inactivation, non-coding RNAs may be part of the building blocks that are necessary to remodel the chromatin architecture on the *Drosophila* X chromosome. The evidence — albeit largely circumstantial — is compelling. Meller *et al.*⁴ and Amrein and Axel⁵ report the existence of two genes, *roX1* and *roX2* (*roX* for RNA on the X chromosome) which, like *Xist*, do not encode proteins, but whose expression is male-specific for most of the life cycle of the fly. Moreover, mutations in the *msl* genes prevent the expression of *roX1* and *roX2*, and ectopic expression (in females) of the normally male-specific *msl-2* gene induces expression of *roX1* and *roX2*.

Whether the *msl* genes regulate *roX* expression at the level of transcription or RNA stabilization remains to be determined. The key piece of circumstantial evidence for the involvement of the *roX* genes in dosage compensation is provided by Meller *et al.*⁴, who show that *roX1* paints the male X chro-

somosome (in a way that is superficially similar to painting of the mammalian inactive X chromosome by *Xist* RNA), and that this localization is dependent on the *msl* genes. Taken together with studies showing that an RNA component is required for association of the MSL complex with the male X chromosome¹², these data indicate that the building blocks needed to remodel the chromatin architecture are RNA-protein complexes. But, as yet, there is no genetic evidence that the *roX* genes are essential for dosage compensation — mutations that eliminate *roX1* have no mutant phenotype. Whether *roX2* alone or the *roX1* and *roX2* RNAs together are necessary for upregulation of the male X chromosome awaits the isolation of *roX2* mutants and, potentially, other *roX* genes.

Superficial similarities aside, there are certainly mechanistic differences between dosage compensation in mammals and flies. For

example, X-chromosome specificity in mammals is probably determined by the *cis*-limited nature of the *Xist* RNA, even in translocations between the X chromosome and autosomes. By contrast, in *Drosophila*, *roX1* RNA associates with portions of the male X chromosome either in *cis* or in *trans*⁴, and X-chromosome specificity is a function of as yet unidentified features of X-chromosome DNA. And, even assuming that the two systems are mechanistically and perhaps evolutionarily related, the biggest question is: how does the RNA-protein chromatin-remodelling complex have such opposite effects in the two systems — hypertranscription in *Drosophila* and gene inactivation in mammals?

In spite of all this, the weight of the evidence indicates that RNA-protein complexes are the key to establishing and/or maintaining dosage compensation in both mammals and flies. The similarities invite one to start thinking about protein partners for *Xist*, to explore the potential functional equivalence of *Xist* and *roX* RNAs, and to predict the possible existence of an RNA component for the nematode system. □

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Condensed-matter physics

New forms of phase segregation

John B. Goodenough and J.-S. Zhou

In the neighbourhood of their ferromagnetic transition temperature T_c , some manganese-oxide perovskites show intrinsic 'colossal' magnetoresistance (CMR) — that is, a dramatic decrease in resistivity with applied magnetic field. Why? On page 256 of this issue¹, De Teresa *et al.* describe how small-angle neutron scattering (SANS) reveals ferromagnetic clusters of manganese atoms, about 12 Å across, that occur within the paramagnetic matrix of $\text{La}_{0.67}\text{Ca}_{0.33}\text{MnO}_3$. These clusters grow and their number decreases with the application of a magnetic field. In addition, between T_c and $1.8T_c$, the material shrinks on cooling

less rapidly than theory predicts, with a sharp, but not discontinuous, return to the theoretical curve on cooling below $T_c = 260$ K. The authors conclude that, in their sample, the CMR is due to the confinement of the charge carriers to finite, ferromagnetic domains in the interval $T_c < T < 1.8T_c$.

Delocalized carriers confined to regions of short-range ferromagnetic order above T_c are called 'magnetic polarons'. They are usually distinguished from localized charge carriers, 'small polarons', confined temporarily to a single cation site by a local lattice deformation because the time needed for a carrier to jump to a neighbouring site is long com-

Superficial relaxation

The thing engineers have nightmares about is brittle fracture. A component carries its maximum tension at its surface, which is covered in stress concentrations of all kinds. A fatal crack may start from the surface long before the bulk of the material is seriously stressed. 'Toughened' glass has its surface put into compression by thermal or chemical treatment. With luck, even severe deformation will not stretch it enough to load the surface in tension and start a crack. Daedalus now has a scheme to relax extremely high surface stresses in engineering materials such as metals.

His plan is to put the material under its design loading, and then heat the surface only. It will anneal and relax, fully shedding all dangerous tensions. The process should work not only on single components, but on whole assemblies: bridges, turbines, aircraft, and so on. There is no need even to know their distributions of stress. But how to heat their surfaces so briefly that the bulk stays cool? Nanosecond laser pulsing should work for small objects. But for big ones, Daedalus proposes surface explosion. Thus iron and picric acid react on contact to give the sensitive detonator iron picrate — which often caused trouble in the days when picric acid explosives were put in iron shells. Many other metals form sensitive explosive compounds — lead azide, copper acetylide, and so on. DREADCO's chemists are now devising intimate explosive coatings for all the engineering metals.

The turbine, plane, or whatever will have its tensile surfaces painted with the explosive reagent mix, and will then be spun or stressed up to the greatest load it can endure. The explosive then will be fired. The superficial blast will race over the tensed surfaces, annealing them in its fierce but momentary passage. The bulk material will receive a compressive shock (which, acting in opposition to the applied tensile loading, will do no harm), but no significant heating. The assembly can then be relaxed. The resulting discoloured or burnt surface must not be smartened up by polishing; that might remove or damage the crucial annealed layer, now in strong compression. Instead, it must be sealed in by paint or plating. Thereafter, the assembly will bear unprecedented loads.

Components such as girders and wire rope, and assemblies such as cars, aircraft and rockets, all will benefit from explosive de-stressing. Even existing installations might, with one mighty bang, be toughened up for heavier duties.

David Jones

pared with the relaxation period of the local lattice deformation that 'dresses' the carrier. These small polarons move diffusively — that is, thermal energy is needed for them to hop from one site to the next.

De Teresa *et al.* suggest that the increase in thermal expansion in their sample in the range $T_c < T < 1.8T_c$ indicates that the ferromagnetic clusters are dressed by cooperative lattice deformations, which would make them 'small' magnetic polarons consisting of several cation centres. The growth in size and decrease in the number of ferromagnetic domains on application of a magnetic field above T_c implies that the field stabilizes greater ferromagnetic order, so these polarons may condense into larger ferromagnetic domains containing many delocalized charge carriers. Within a ferromagnetic domain, the conductivity is presumably metallic, so the resistance decreases as the fraction of the metallic volume increases.

Aside from their technological potential, the manganese oxides are important because, like the copper-oxide superconductors, they appear to exemplify a dynamic phase segregation occurring at a transition from localized to itinerant electronic behaviour. Where there is a discontinuous increase in the mean kinetic energy of the electrons, there must be a corresponding decrease in their mean potential energy according to the virial theorem of classical mechanics. A jump in potential energy would be reflected in a discontinuous change in the equilibrium metal-oxygen bond length and hence in the volume at the transition, making this a first-order transition.

First-order transitions may give rise to either a global, homogeneous change of volume or a phase segregation. Phase segregation is normally accomplished by atomic diffusion, which can only occur at higher temperatures. But at a transition from localized to itinerant electronic behaviour, phase segregation may be accomplished at low temperatures by cooperative atomic displacements that create domains of shorter bond lengths containing delocalized electrons, separated by regions where the atoms contain localized electrons. Such cooperative atomic displacements may either be static or remain dynamic down to the lowest temperatures.

In the $\text{La}_{1-x}\text{A}_x\text{MnO}_3$ perovskites, the transition from localized to itinerant (delocalized) behaviour of the conduction electrons occurs in the presence of localized electron configurations having a total localized spin $S = 3/2$, so the phase containing mobile charge carriers is ferromagnetic, whereas the phase with no delocalized charge carriers remains paramagnetic in the range $T_c \leq T \leq 1.8T_c$ in $\text{La}_{0.67}\text{Ca}_{0.33}\text{MnO}_3$. Where a dynamic phase segregation occurs, as appears to happen in this case, application of a magnetic field above T_c stabilizes the ferromagnetic relative to the paramagnetic phase, which increases the volume fraction of the

more conductive ferromagnetic phase to give a colossal magnetoresistance.

Oxides with perovskite-related structures can accomplish a dynamic phase segregation by moving their oxygen atoms without having to make a large perturbation of the cation positions. If these displacements are dynamic or spatially aperiodic, the heterogeneity introduced by the phase segregation cannot be detected by a diffraction experiment, and so some other technique is required to reveal it. Small-angle neutron scattering can detect a magnetic heterogeneity involving larger ferromagnetic clusters in a paramagnetic background, but not clusters involving only two or three manganese atoms.

The magnetic susceptibility data of De Teresa *et al.* indicate the presence, at temperatures $> 1.8T_c$, of superparamagnetic clusters consisting of ferromagnetically coupled $\text{Mn(III)} + \text{Mn(IV)}$ pairs. These Zener³ pairs become segregated below $1.8T_c$ into larger ferromagnetic regions within a paramagnetic matrix. Zener pairs are charge carriers, and their segregation into isolated ferromagnetic regions traps them, but they are released by cooling below T_c or by a magnetic field that makes the ferromagnetic regions grow until they overlap. De Teresa *et al.* assume that the conduction electrons become itinerant below T_c because they observe long-range magnetic order and a low resistivity. However, the localized-electron phase would also become ferromagnetic below the long-range ordering temperature T_c , so the disappearance of magnetic heterogeneity does not guarantee the disappearance of small mobile clusters. The resistivity below T_c remains high relative to that expected for a conventional metal, and Louca *et al.*² have identified three-atom dynamic clusters at 12 K, well below T_c , which is out of the detection range of SANS. It appears that De Teresa *et al.* may have identified a trapping out of Zener-pair charge carriers in larger ferromagnetic clusters in the range $T_c < T < 1.8T_c$.

It is now becoming more generally recognized that the copper-oxide superconductors may owe their peculiar transport properties to a similar dynamic phase segregation. Non-adiabatic polarons containing just five or six copper centres⁴ in underdoped oxides condense into large 'stripe' domains^{5,6} in the more heavily doped, superconducting compositions. The realization that such a dynamic phase segregation can occur in real systems is opening a new chapter in solid-state science, and is providing surprises that may yet prove to have great technical importance. □

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A hormonal mechanism for parental favouritism

In many taxa, parents compensate for the overproduction of young by conferring vital advantages on certain offspring, enabling them to out-compete their siblings when resources are scarce^{1,2}. With birds, the competitive advantage is usually related to the age of chicks, which varies because of earlier hatching and incubation²⁻⁴. Recent studies of canaries (*Serinus canaria*) show that hen birds may influence competitiveness directly by depositing increasing amounts of testosterone in the yolks of successively laid eggs⁵⁻⁷. We have observed a contrasting phenomenon in cattle egrets (*Bubulcus ibis*), where egret mothers deposit more androgens in the first eggs of their clutches, adding a potential hormonal boost to the advantage caused by earlier hatching. This may help senior siblings eliminate junior nest-mates.

The higher doses of testosterone bestowed by canary mothers on later-laid chicks^{3,6} increases their begging vigour⁷. This confers growth advantages when chicks hatch at the same time, but does not fully compensate for the disadvantages of hatching late in asynchronous broods. The effects of incubation onset and testosterone dosage work in opposition, so canary mothers can favour either their early-egg offspring (by early hatching) or those from later-laid eggs (by testosterone in tandem with synchronous hatching)⁷. Comparative studies were needed to assess the generality of such hormonal influences on sibling competition⁸.

Cattle egrets offer an ideal contrast to canaries. The separation of hatching times produces large differences in the size and strength of siblings, reducing the number of fights between nestlings and aiding the elimination of junior brood members during food shortages (siblicide)⁹. It would be surprising if senior egret chicks were empowered by the earlier hatching, at the

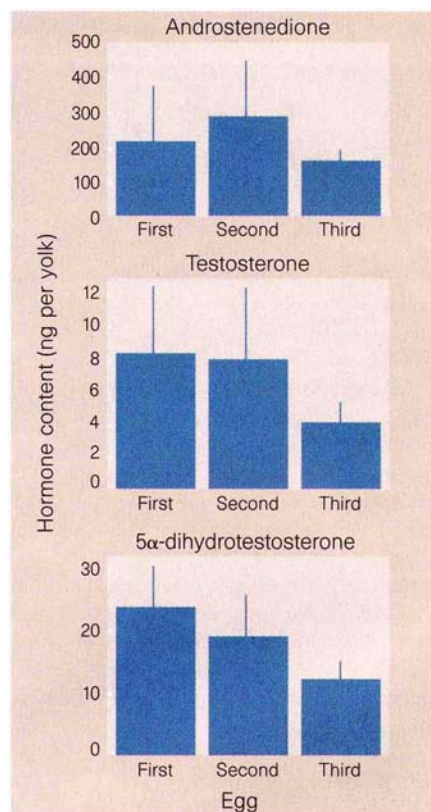


Figure 1 Mean yolk contents (error bars show 95% confidence limits) of maternal androgens androstenedione, testosterone and 5 α -dihydrotestosterone for first-, second- and third-laid cattle egret eggs in a clutch. Repeated-measures ANOVA showed significant differences in all three hormone levels between chicks (androstenedione, $P = 0.0359$; testosterone, $P = 0.0022$; 5 α -dihydrotestosterone, $P = 0.008$).

same time as being hormonally handicapped by low yolk androgen levels.

Our studies of eight three-egg cattle egret clutches (the modal clutch size), each egg collected on the day it was laid, show that

the two first-laid eggs contain more androgens than the third egg (Fig. 1). This suggests that, in contrast to canaries, cattle egret androgens act in concert with hatching asynchrony to favour senior siblings. We do not know whether these hormones influence the aggression or begging of egret chicks, but experimental manipulations of yolk testosterone levels in canaries show that androgens enhance begging behaviour⁷, growth rate⁷ and aggression⁵. Our results suggest that hormonal favouritism may be a common mechanism for bird mothers to influence sibling competitiveness. They also support the view that parents are not in evolutionary conflict with siblicidal offspring, but may be subtle participants in the process of orderly brood reduction¹⁰.

These findings underscore the need for cross-taxonomic surveys of yolk androgens in relation to laying order, sibling rivalry and reproductive strategy⁸, and for within-species studies of broods facing different conditions⁶, to elucidate the function of maternal hormones in reproductive optimization. In turn, this should illuminate how 'trans-generational' hormone effects¹¹ and the evolutionary interests of parents² have helped to shape the endocrine control of reproductive systems.

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Self-recognition by proteoglycans

During the emergence of multicellular organisms, molecular mechanisms have arisen to allow self-recognition and discrimination against 'non-self'. We suggest that cell-surface proteoglycans might have provided these key recognition and adhesion functions. If so, the simplest Metazoans alive today, such as Porifera

(sponges), should retain, at least in part, proteoglycan adhesion and recognition mechanisms. We have shown, using atomic force microscopy, that proteoglycan-to-proteoglycan binding produces fundamental cell cohesion forces in the sponge *Microciona prolifera*¹, as previously implied by functional investigations^{2,3}. Early work on cell adhesion of dissociated marine sponge cells provided evidence for cell sorting by species²⁻⁴. Here we show that proteoglycans underlie the molecular mechanism of this self-recognition.

We mixed metabolically attenuated cells (at 0 °C) of three marine sponge species, *Microciona prolifera*, *Halichondria panicea* and *Cliona celata*, bearing surface proteoglycans, in artificial sea water. Within 5–15 min species-specific recognition and adhesion occurred, but only when the sea water contained a physiological concentration of Ca²⁺ (Fig. 1a). After selective washing of proteoglycans from the cell surface, none of the three species displayed aggregation. Replacing the purified proteoglycans (again at 0 °C) completely restored species-

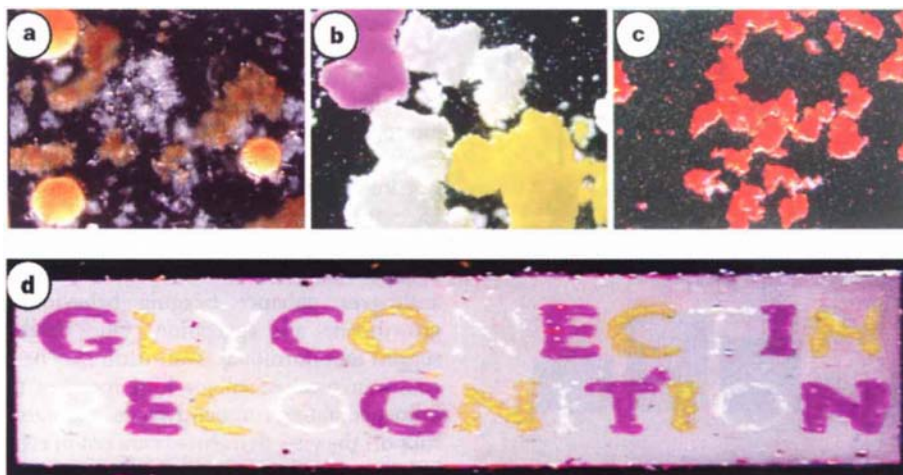


Figure 1 a, Discrimination between self and non-self, in a suspension of live glyconectin-bearing *M. prolifera* (orange), *H. panicea* (white) and *C. celata* (brown) sponge cells, at 0 °C. The microscopically observed colours are different from the whole sponge. b, Ca^{2+} -dependent species-specific recognition and adhesion of pink, yellow and white beads coated with glyconectins 1, 2 and 3, respectively. c, Control experiment showing a lack of sorting of pink, yellow and white beads, all coated with glyconectin 1. Magnification $\times 40$. d, Species-specific glyconectin-to-glyconectin recognition in a blotting assay. Letters were drawn using glyconectins ($4 \mu\text{l}$ at $1.5 \text{ g } \mu\text{l}^{-1}$) on a nitrocellulose membrane (Hybond-C extra) and probed with glyconectin-coated beads. Photographs taken after 30 min constant mixing.

selective cohesion, showing that specific cell-surface proteoglycans and Ca^{2+} are essential for cell recognition and adhesion of these sponges.

Biochemical analysis of isolated proteoglycans showed distinct carbohydrate structures that are different from classical mammalian proteoglycans^{3,5,6}, suggesting that sponge macromolecules define a new class of primordial proteoglycans. We have named the proteoglycans glyconectins 1 (*M. prolifera*), 2 (*H. panicea*) and 3 (*C. celata*). To test whether glyconectins 2 and 3 exhibit the same calcium-dependent self-adhesion as glyconectin 1 and to measure the specificity of glyconectin–glyconectin interactions, we developed a colour-coded bead-adhesion system.

We attached glyconectin 1 to pink, glyconectin 2 to yellow, and glyconectin 3 to white latex-amidine beads, and mixed the three bead types in artificial sea water. Again within 5–15 min, species-specific bead aggregation occurred (Fig. 1b). Control experiments with glyconectin 1 attached to pink, yellow and white beads resulted in mixed, orange-coloured aggregates, as expected (Fig. 1c). In the absence of Ca^{2+} , bead aggregation did not occur.

Specificity of glyconectin–glyconectin binding was further demonstrated by incubating glyconectins immobilized on a nitrocellulose membrane with the glyconectin-coated beads. The three molecules were used to draw the letters of the words 'glyconectin recognition'. Homophilic recognition between membrane-bound and bead glyconectins was evident from the selective staining of each letter

(Fig. 1d). This simple bead assay, which mimics interactions between sessile and free cellular forms in a primeval environment, shows that glyconectins could enable discrimination between self and non-self.

The specificity of homophilic glyconectin interactions in Porifera approaches the degree of selectivity of the evolutionarily advanced heterophilic immunoglobulin superfamily recognition system. Glyconectin–glyconectin interactions may thus provide a new model for molecular self-recognition. We propose that the evolution of glyconectin-like proteoglycan molecules, with the capacity for self-recognition and adhesion, may have been a fundamental requirement for the establishment of the first multicellular organisms, as well as for the further divergence of species, and the appearance of more complex multicellular forms of life.

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Typing prion isoforms

The central event in the pathogenesis of prion diseases is the conversion of the cellular prion protein (PrP^{C}) into the pathological and protease-resistant isoform (PrP^{res}). The conversion produces PrP^{res} types with distinct physical and chemical characteristics^{1–4}, which may represent the molecular basis for prion strains^{1,2,4}. Collinge *et al.*³ extended our original observation of two types of PrP^{res} in sporadic Creutzfeldt–Jakob disease (sCJD)², into iatrogenic CJD (iCJD) and the so-called new variant CJD (vCJD)^{5,6}, which may have been acquired from bovine spongiform encephalopathy (BSE). On the basis of electrophoretic mobilities and ratios of differently glycosylated isoforms, Collinge *et al.* reported³ a total of four types of PrP^{res} , but our analysis of a large series of additional cases including kuru (associated with cannibalism in the Fore tribe of New Guinea)⁷, iCJD and vCJD reveals only two patterns of electrophoretic mobility as we originally reported² (Table 1).

We performed immunoblot analysis on brain homogenate samples from a total of 97 human subjects with non-inherited prion diseases. They included 88 cases of sCJD, six of iCJD, one of vCJD and two of kuru (Table 1). We confirmed the presence of two PrP^{res} types, with electrophoretic mobilities corresponding to relative molecular masses (M_r) of $\sim 21,000$ (type 1) and $\sim 19,000$ (type 2), in the sCJD cases (Table 1). The five iCJD PrP^{res} samples from subjects homozygous for methionine at codon 129 comigrated with PrP^{res} type 1, whereas the sample from the one subject homozygous for valine comigrated with PrP^{res} type 2, as did the samples from the kuru and vCJD subjects (Fig. 1 and Table 1).

Collinge *et al.* observed³ PrP^{res} type 1 and 2 in sCJD subjects and in iCJD subjects homozygous for methionine at codon 129. Type 3 was seen exclusively in iCJD subjects carrying at least one valine allele at codon 129 who acquired the disease through contaminated pituitary-derived hormones. The PrP^{res} associated with vCJD was found to have the same gel mobility as type 3 but was separated as type 4 on the basis of a distinct ratio of the three glycosylated isoforms.

Our results suggest that Collinge *et al.* may have erroneously matched our sCJD type 1 and 2 with theirs. We believe that most, or all, of the type 1 and 2 cases identified by them actually correspond to our type 1, whereas their type 3 matches our type 2. Indeed, they found a higher frequency of sCJD type 2 than of sCJD type 1, whereas we linked PrP^{res} type 1 to the most common sCJD phenotype. Moreover, 13 of their 18 sCJD cases homozygous for methionine at *PRNP* codon 129 had the

Table 1 Molecular features of the CJD and kuru subjects

Disease classification	Codon 129 genotype	PrP ^{res} type 1	PrP ^{res} type 2A	PrP ^{res} type 2B
Sporadic	M/M	54	4	
Sporadic	M/V	5	7	
Sporadic	V/V	3	15	
Iatrogenic (growth hormone)	M/M	1		
Iatrogenic ^a (growth hormone)	V/V		1	
Iatrogenic (dura mater)	M/M	2		
Iatrogenic (corneal)	M/M	1		
Iatrogenic (electrodes)	M/M	1		
Kuru	M/V		1	
Kuru	V/V		1	
New variant ^a	M/M			1

The number of cases of PrP^{res} types 1 and 2, for the listed disease types, are given. M, methionine; V, valine.

PrP^{res} type 2. This would indicate that more than half of their subjects belong to this group, whereas in our series (in which 65% of subjects were European) this variant only accounts for about 5% of cases.

In our analysis of virtually all forms of CJD⁸, we found a small variability in the molecular mass of PrP^{res} type 1, which showed no obvious correlation with the disease phenotype. This variability may account for the difference in M_r between the PrP^{res} type 1 and 2 of Collinge *et al.* If all of the sCJD cases examined carried PrP^{res} type 1, then Collinge *et al.* would have observed PrP^{res} type 1 in all codon-129 genotypes, at variance with our previous report but consistent with our more recent data (Table 1) showing PrP^{res} type 1 in sCJD-affected sub-

jects that carry the codon 129 valine allele.

Glycosylation analysis confirms the finding³ that in vCJD the high- M_r glycoform is the most abundant, and relatively little unglycosylated PrP is present — a pattern seen in familial subtypes of CJD⁸, but not observed in our other groups (Fig. 1c).

The acquired forms of human prion disease such as iCJD and kuru reproduce the patterns of PrP^{res} seen in sCJD, indicating that PrP^{res} characteristics might be maintained after human-human transmission, irrespective of the route of infection. vCJD shares the PrP^{res} electrophoretic mobility with a subset of sCJD, leaving only the glycoform difference to discriminate vCJD PrP^{res} from sCJD and iCJD PrP^{res}. Although the glycoform ratio seems to be a valuable

molecular marker for the diagnosis of vCJD, we question the wisdom of identifying prion strain types in different animal species solely on the basis of PrP^{res} glycoform pattern to identify of the prion strain that causes BSE and vCJD. Glycosylation is a co- and post-translational event likely to be affected by the cell type of the species in which it occurs. Indeed, transmission of sCJD type 1 to chimaeric transgenic animals consistently reproduces the size but not the glycoform pattern of the original PrP^{res} inoculate (ref. 4 and unpublished data).

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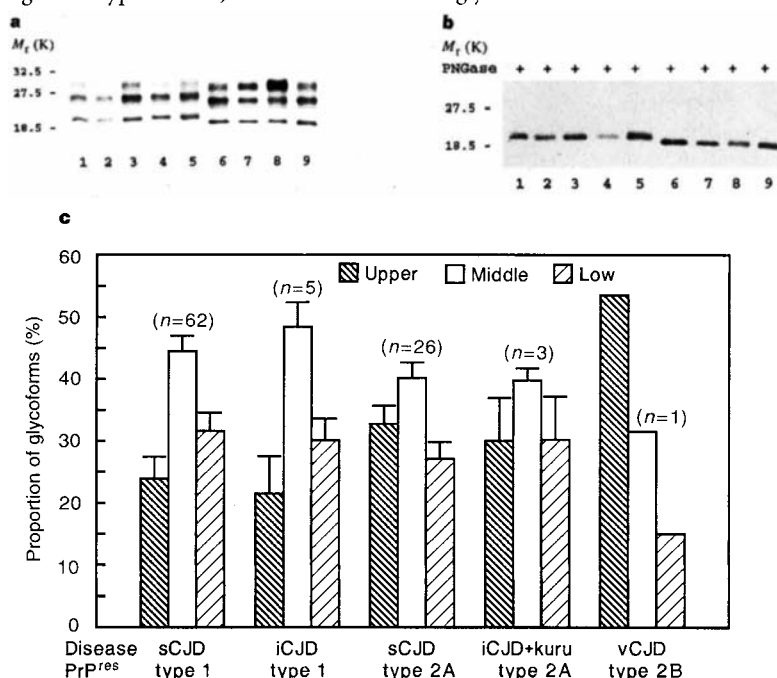


Figure 1 Western blot of proteinase K-treated brain homogenates from subjects with sCJD, iCJD, vCJD and kuru, before (a) and after (b) deglycosylation. All samples were obtained from the cerebral cortex. The anti-PrP monoclonal antibody 3F4 was used. Lanes 1–5, PrP^{res} type 1; lanes 6–9 PrP^{res} type 2; lanes 1, sCJD, codon 129 M/M; lanes 2, iCJD (dura mater), codon 129 M/M; lanes 3, iCJD (electrodes), codon 129 M/M; lanes 4, iCJD (corneal), codon 129 M/M; lanes 5, iCJD (growth hormone), codon 129 M/M; lanes 6, sCJD, codon 129 V/V; lanes 7, iCJD, codon 129 V/V; lanes 8, vCJD, codon 129 M/M; lanes 9, kuru, codon 129 V/V. c, Relative proportion of different PrP^{res} glycoforms in sCJD, acquired CJD and vCJD. Mean $\pm$ s.d. Upper, high-molecular-mass glycoform; middle, low-molecular-mass glycoform; low, unglycosylated form. PrP^{res} type 2 A and B have a distinct ratio of glycoforms.

Collinge *et al.* reply — We recently reported four different protease-resistant PrP patterns from sporadic, iatrogenic and new variant CJD patients³. Parchi *et al.* have extended their original study of sporadic CJD¹⁰, in which two PrP^{Sc} types were demonstrated, and include both a number of acquired prion diseases (iatrogenic CJD and kuru) and a single new variant CJD case. They also confirm our finding that new variant CJD can be distinguished on western blots from sporadic and iatrogenic CJD by differences in the relative intensities of the three PrP glycoforms, which we designated a 'type 4' pattern.

For iatrogenic CJD, however, they found only their original two patterns. We reported a 'type 3' pattern in iatrogenic CJD cases arising from treatment with hormones derived from post-mortem pituitaries which were of genotype M/V or V/V at polymorphic PrP residue 129. Their single such case (of genotype V/V) was found to be of their type 2 pattern. In more extensive studies of iatrogenic CJD we have found that most, but not all, such cases are type 3. It is possible, therefore, that this iatrogenic CJD case may simply not have been 'type 3'.

It may be premature to draw conclusions on the basis of a single case. Furthermore, the suggestion that all our 'type 3' cases are in fact what Gambetti and colleagues classify as 'type 2' does not solve the apparent discrepancy between the data sets. We would

then not have found Parchi's type 2 in sporadic CJD at all, whereas he finds this type in around 30% of cases. There are clear differences in western blotting methods between the two studies that may be important in detecting the relevant band shifts. However, our finding of four distinct patterns has been independently confirmed in a recent study of French CJD cases, which included the same new variant CJD that Parchi *et al.* studied¹¹.

As we reported³, there seems to be further heterogeneity within the four PrP^{Sc} types, and it seems likely that this will be only a first approximation towards a molecular classification of human prion diseases. The techniques used to date are relatively crude and relate to use of proteinase K alone. Higher resolution methods to size fragments, and the use of other proteases may detect yet further heterogeneity. In our view, both studies should be followed up with much larger-scale analyses in an attempt to relate PrP^{Sc} types to clinicopathological phenotypes in humans and to study their transmission characteristics in susceptible animals. It will be important to standardize PrP^{Sc} typing methods between centres with exchanges of sample sets to allow definition of an agreed classification of PrP^{Sc} types.

For PrP conformation, or any other molecular candidate, to provide a basis for encoding prion strain specificity, it must be transmissible on passage to laboratory animals. That the PrP^{Sc} types we have described in sporadic and iatrogenic CJD are maintained on passage of prions to transgenic mice expressing wild-type human PrP argues that the preliminary classification of PrP^{Sc} types we have proposed has biological relevance³.

Finally, we did not claim the 'identity' of the prion strain causing BSE and vCJD. We reported that the glycoform pattern of vCJD differed significantly from other forms of CJD (as Parchi *et al.* confirm) but resembled that seen in BSE in cattle and BSE transmitted to several other species, consistent with, but not proving, the hypothesis that they are causally related³.

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Medaka fish for mutant screens

The great success achieved by the groups led by Janni Nusslein-Volhard and Wolfgang Driever has undoubtedly established the zebrafish as a most promising model for vertebrate developmental genetics. But, we would like to champion the Japanese medaka (*Oryzias latipes*) as an equally useful experimental model.

Holder and McMahon¹ pointed out that the medaka was not used by the late George Streisinger, an originator of zebrafish developmental genetics, because it is difficult to score embryos for a phenotype in the clustered eggs of medaka. It is in fact quite easy to observe embryonic phenotypes in the medaka. The medaka lays a cluster of eggs every day. The entire cluster can be isolated from the mother using a net, without losing any sib embryos. Single eggs, which have hard chorions, can be isolated by rubbing the cluster between two small pieces of paper towel. The procedure is simple, and single eggs can be obtained within seconds. The embryos and chorions are transparent, and thus the phenotype can be scored in the medaka just as it is in the zebrafish.

In the medaka, more than 80 spontaneous visible mutants including about 30 morphological mutants have become available for experimental work and are currently maintained at Nagoya University². The generation of developmental mutants of the medaka by exposure to radiation and chemicals was also reported recently³. Developmental mutants such as *Da* (double anal fin), *el* (eyeless), *fu* (fused vertebrae), *pl* (pectoral finless) and *tb* (twisted brain) are important resources for studying vertebrate development.

The medaka has been used by many investigators, including our group, in developmental and genetic studies for more than 70 years. Several inbred strains of the medaka have become available for experimental work⁴ and a detailed genetic map is now also available⁵. To produce knockout fish for the analysis of gene functions, medaka embryonic stem cell lines have been established⁶. Thus the medaka, like the zebrafish, is a suitable species for vertebrate developmental genetics studies.

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Recent information on studies using medaka is available at the Internet 'Medakafish Homepage' (<http://biol1.bio.nagoya-u.ac.jp:8000/>).

Natural selection bias?

Alatalo *et al.* in their Scientific Correspondence¹ suggest that an increase in published heritability estimates since 1988 results from a paradigm shift, increasing willingness to publish such estimates based on small sample sizes. But there have been several changes in the types of studies published since 1988 which may also contribute to this observation.

Most striking has been the increase in examination of sexual selection in birds. Before 1988, of the ten studies cited in ref. 2, nine are on insects, and one is on fish. After 1988, nine of twenty-four are studies of birds. This goes some way to explaining the decrease in sample size observed by Alatalo *et al.*, four out of seven of which are bird studies whereas only one is on insects. It tends to be easier to amass large data sets using insects, so referees may be less likely to accept low sample sizes in these cases.

A second change has been in the type of study carried out. Before 1988, seven of eleven studies were based on artificial selection. Since 1988 there has been a marked increase in studies using parent-offspring or sib-sib regression in the wild (nine of twenty-four since 1988 as opposed to one of eleven previously).

These changes suggest a more pernicious explanation for the proposed kuhnian shift. Perhaps it is not publication bias, but a bias in the design of studies carried out. Scientists cannot risk (or gain funding for) research without a high probability that a 'successful' result will be obtained. Having been convinced of the credibility of the good-genes theory, could it be possible that researchers have felt more inclined to conduct heritability studies on systems that they believe will yield orthodox results?

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A night at the operon

The *lac* Operon: A Short History of a Genetic Paradigm

by Benno Müller-Hill

Walter de Gruyter: 1996. Pp. 207. \$35.95, DM54

Sydney Brenner

This book opens with the lament that for young molecular biologists history does not exist, and that they have no interest in the long struggle that has made the subject what it is today. I hold the somewhat weaker view that history does exist for the young, but is divided into two epochs: the past two years, and everything that went before. That these have equal weight is a reflection of the exponential growth of the subject, and the urgent need to possess the future and acquire it more rapidly than anybody else does not make for empathy with the past.

Benno Müller-Hill says that "one has to grow old to understand the functioning of a science". He has written a history of the *lac* system of *Escherichia coli*. He warns us that it is not a definitive history, which comes as a relief because that kind of history would have been boring. What Müller-Hill has written is partly historical, partly autobiographical, and even partly philosophical. It is best described as a collection of stories (with a few parables, too) disguised as a history of what is now a small and almost unoccupied corner of molecular biology.

About 30 years ago the *lac* operon stood in the centre of the scientific stage, and provided the experimental basis for discovering the principles of the control of gene expression. Parallel research on the *lac* operon and bacteriophage λ formed the basis for the operon theory of gene regulation put forward by François Jacob and Jacques Monod in 1961. One central idea was that regulation was negative, that is, if the genes were placed in a control vacuum they would be unleashed and express as much as they could. Their expression was held back by repressors, and they were turned on by the removal of repression, either by an inducer in the case of the *lac* genes, or by destruction of the repressor in bacteriophage λ . Repressors were later found that required a small molecule to make them active so they could turn off their target genes.

So central was this idea of negative control that it became established doctrine, and those few scientists who worked on positive regulation were dismissed and had to struggle for a hearing. It was later found that cap factor acts positively on the *lac* operon, and that the λ repressor exerts a positive effect on its own synthesis, so the dogma of negative control gradually disappeared.

Evidence for the theory came from the

The three winners: François Jacob, Andre Lwoff and Jacques Monod receiving the 1965 Nobel Prize for Physiology or Medicine for their research into the genetic control of enzyme activity.

discovery of operator-constitutive mutants, which were *cis* dominant, affecting the regulation of both β -galactosidase and lactose permease on the same chromosome. The realization that virulent mutants of bacteriophage λ were equivalent reinforced the conclusion that they had to affect a site on the DNA that interacted with the repressor and controlled the expression of two different genes. Operator-zero mutants also acted in *cis*, but these were later shown to be due to nonsense mutants with strong polarity; they had nothing to do with operators but nevertheless required the idea of the operon, a unit of expression containing more than one gene.

Müller-Hill and Walter Gilbert succeeded in isolating the *lac* repressor in 1966. This was quite an achievement, as the only assay for this repressor was that it bound the inducer, IPTG, and calculations showed that the value of the binding constant and the number of repressor molecules would not allow detection by equilibrium dialysis. Müller-Hill selected for a mutant that had a higher affinity for the inducer than the wild-type repressor and this tight-binding mutant made the experiments feasible.

The contemporary student finds it hard to understand that this research always had to be carried out by indirect means, and not by looking directly at genes as we do today. The design and interpretation of such experiments was the core skill of molecular biology, and critical experiments often resorted to what Francis Crick once called "special genetic tricks", a primitive kind of genetic engineering. It was also important to ensure that theories did more than explain the facts

on which they were based, and to distinguish prediction from retrodiction.

The move to research on eukaryotes began at the end of the 1960s, and prokaryotic research started to decline. We argued for many years about whether the Jacob-Monod model would have anything to do with cell differentiation in higher organisms, and it is ironic that positive regulation is generally found, with many transcription factors acting to enhance gene expression. There was disappointment at the general absence of operons and the resulting unitary control of a group of genes by one operator. What has survived, though, is the central principle that regulation is mediated by specific recognition of DNA sequences by proteins.

When molecular biology finally comes to an end, and a massive crash of the computer system in the library at Alexandria corrupts most knowledge, I hope that historians of science will unearth a copy of this book. They will surely debate whether all of the stories are true and whether they could have been written by the same person, and might even suggest that Müller-Hill was at least two people. What will certainly puzzle them is that it is full of characters they have never encountered before, and that even the famous ones are hard to recognize. The only thing they will learn about Watson and Crick is that the former produced a naked lady for the latter's fiftieth birthday. Was this perhaps part of some obscure symbolic ritual enacted each year to celebrate the birth of another model? □

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Van der Waals: known for his forces.

A force in physical chemistry

Van der Waals and Molecular Science

by A. Ya. Kipnis, B. E. Yavelov and J. S. Rowlinson

Oxford University Press: 1996.

Pp. 313. £60, \$105

Charles Tanford and Jacqueline Reynolds

Theoretical physical chemistry is difficult. It strives to emulate the virtues of theoretical physics — conceptual clarity and mathematical rigour — but must also make provision for the individual characteristics of every chemical substance to which its equations may be applied. Compromise is inevitable, often leading to the inclusion of parameters hopelessly beyond our ability to calculate or measure. The most notorious of these are the dimensionless ‘activity coefficients’ introduced by Gilbert Newton Lewis, which could mean (mechanistically) whatever the user wanted them to mean.

Johannes Diderik van der Waals (1837–1923) was a mathematician and physicist who devoted his life to one of the central problems in physical chemistry — the behaviour of real gases and condensed phases and the transition between the two. His

familiar equation of state formed part of his doctoral thesis, presented to the faculty at the University of Leiden in 1873 and described in the book as “the great event of his life”. His equation is clearly a step above Lewis’s extreme empiricism, and his parameters have a qualitatively clear meaning and appropriate dimensions: ‘van der Waals forces’ and ‘van der Waals radius’ are household words to physical chemists to this day. His derivation, although not as rigorous as one might like, contributed greatly to the understanding of molecular events and led to the idea of continuity between gaseous and liquid states. He received a belated Nobel prize for physics in 1910, long after his seminal work was actually done.

Van der Waals and Molecular Science was originally published in 1985 in Russian, by Kipnis and Yavelov alone. The present version is a translation, with J. S. Rowlinson as a third author. There are two new chapters, which admirably and succinctly summarize modern concepts in the field and relate them to van der Waals’ more “pictorial” views of 120 years ago.

The main body of the text is less well focused than the added chapters. It is neither a biography in the strictest meaning of the word nor a history of the development of the field of non-ideal gases and solutions, but falls somewhere between the two. An attempt is made to provide a picture of contemporary science and science education in the late nineteenth century, with an emphasis on Dutch science; this includes perhaps more detail than one might want about van der Waals’ colleagues in the Netherlands. Characters and their associated biographical material are frequently inserted willy-nilly in the text, disrupting the principal story line and contributing little to the understanding of either the man or his ideas.

One interesting feature is a lengthy chapter on Russian reactions to van der Waals and his work, affording a rare glimpse into the Russian scientific scene around the turn of the century. Names unfamiliar to English or American readers abound and we learn about much that is curious, an example being the election of van der Waals (who never visited Russia) as an honorary member of the Imperial St Vladimir University in Kiev in 1902, on the occasion of the twenty-fifth anniversary of his Amsterdam professorship.

Most of the book (and especially the two new chapters) is highly mathematical and will be hard going for the general reader. It will be a useful reference for the specialist in molecular physics because of its somewhat unconventional Dutch and Russian frame of reference, but is not sufficiently comprehensive to be considered definitive for the history of the subject as a whole. □

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Plagues past and present

Yellow Fever Black Goddess: The Coevolution of People and Plagues

by Christopher Wills

Addison-Wesley: 1996. Pp. 318. \$24

Virus X: Understanding the Real Threat of the New Pandemic Plagues

by Frank Ryan

Harper Collins: 1996. Pp. 388. £20/Little Brown \$24.95

Valerius Geist

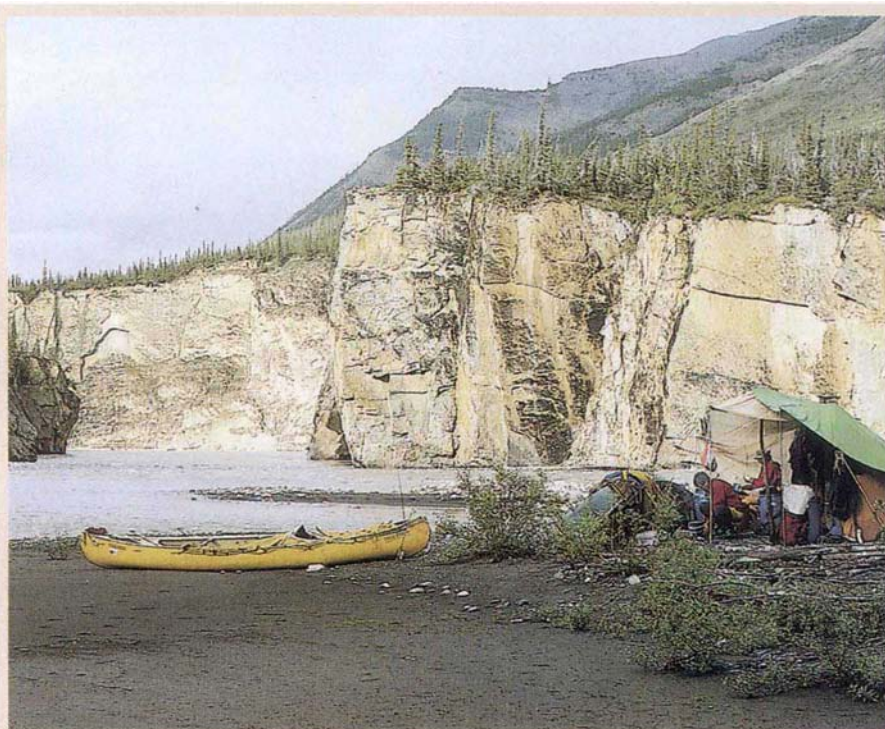
Plagues are not nature’s “solution” to the problem of human overpopulation, writes Christopher Wills. Like it or not, we shall have to address that challenge quite independently of plagues. Wills is more optimistic than Frank Ryan about our ability now and in the future to deal with plagues.

The history and nature of plagues is the subject of both authors’ books, although Ryan’s is a little narrower in scope, dealing primarily with emerging viral diseases. Beyond that the similarities end. Wills’s book is reminiscent of Hans Zinsser’s famous *Rats, Lice and History* (Little Brown, 1935) — it is to the point, scholarly, disinterested and rich in technical detail. In contrast, Ryan’s is written like a detective thriller, emphasizing the human element in hunting down plagues.

Wills’s work is eminently suitable as supplementary reading for introductory courses on evolution, human biology, science in human affairs, environmental science and related subjects. Wills writes like a seasoned professor instructing students. His style is pleasantly chatty, with ample war stories and anecdotes to make his points. It is the better book in imparting and explaining technical information; his treatment of AIDS is masterly. At the same time his historical examples illustrate science as a human endeavour with shortcomings and triumphs. Scientists do not appear as magicians.

Ryan’s book should have greater appeal to non-scientists and, if widely read, could become a significant instrument in policy formation. It is a good volume for managers and planners, as it entertains and binds the reader with its “who did it?” spell, while imparting hard knowledge. It is difficult to put down.

Besides being spellbinding, Ryan’s book is intellectually the more adventurous. It appears that Ryan, immersed in current knowledge about viruses, began to see a greater, strategic vision, though one not necessarily shared by the busy specialists and harassed practitioners whose attention was consumed by the daily grind at the tactical



Up this creek with a paddle

Many parts of the world, including Mountain River in Canada's Northwest Territories (above), are best explored by canoe. But camping in the great outdoors can be a dangerous experience if you're

unprepared. You need to know how to survive. Fortunately, Bill Mason has provided plenty of advice in his book *Song of the Paddle: An Illustrated Guide to Wilderness Camping* (Firefly, \$19.95).

level. I wish that he had had more success in this endeavour. Inadvertently, he makes a very good case for viruses as chromosomal parasites. Students of parasites will find much of the behaviour of viruses comfortably familiar. But Ryan argues that viruses, coevolving with their hosts, do not form parasitic or even commensal relationships, but symbiotic ones. That is, viruses evolve not as accidental but as deliberate protectors of their host species, generating resistance against other viruses while infecting and killing competitors that threaten their host. Although dramatic, this thesis contains less than meets the eye.

Also, Ryan's "genomic intelligence" is not original, being known otherwise as epigenetics. Nor is invoking Gaia and holism, without tracing causative links, an aid to explanation. By missing the point that parasitism is the essence of viruses, Ryan fails to see characteristics of some viruses that would make any good parasite, had it the ability, wince in envy. Imagine a parasite that invades germ-cell chromosomes and is then transmitted with each conception to become a part of each cell of the new host. One cannot do better! In so doing, would there not be an additional cost of replication? If so, then under resource-poor conditions efficiency selection should progressively miniaturize, nay dismember, these parasites. That would

lead to chromosomes peppered with remnants of viruses — as is found.

As to symbionts defending their host, as opposed to parasites accidentally infecting and disabling potential host competitors, how does one adjudicate between these alternatives? An argument for the former cannot be made by listing more and more examples. It begs the question why do competitors not retaliate with their "death viruses"? Theoretically, one expects coevolution between the host-competitors, including resistance to each other's parasites. If so, deadly cross-infections are expected only when host-competitors meet that have never met before, which is what the evidence suggests. Had the study of parasitism identified good examples of how parasites systematically act to infect host-competitors when their host is in need, then the case for symbiosis would have been made and accepted long ago. Moreover, a species carrying loads of deadly parasites need not benefit from cross-infections.

There is hardly a species better equipped with parasites to inflict havoc on competitors than the ancient white-tailed deer. Its collection of parasites dangerous to other deer species include the meningeal worm, giant liver fluke and deer ticks, as well as arboviruses and bacterial and protozoan diseases too many to list. Now,

transmissible spongiform encephalopathy, an echo of 'mad cow disease', has been detected in free-living white-tails. This immense battery of "protectors" strikes down deer species that hardly compete with white-tails, such as caribou and moose, but is of no help when white-tailed deer face effective competitors such as sika, roe or red deer. Transplanted white-tailed deer facing these competitors in Europe and New Zealand have done poorly, while feral sika deer quickly out-compete native white-tailed deer in North America.

Moreover, when colonizing new areas, white-tailed deer tend to shed their parasites and so come in contact with potential competitors just when they would need their "protectors" most. Even their collection of arboviruses (eastern-, western-, St Louis viral encephalitis, and so on) has yet to be recorded as helpful.

Be that as it may, both books provide pleasure and stimulation. □

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Bodies in mind

Being There: Putting Brain, Body, and World Together Again

by Andy Clark

MIT Press: 1997. Pp. 269. \$25, £19.95

Margaret A. Boden

From time to time, ideas that have lain near-dormant, scattered across various fields of research, are revived in a newly integrated approach. The recent integration described by Andy Clark is an approach to cognitive science that stresses "active, embodied, cognition". It includes work in cognitive and developmental psychology, neuroscience, artificial intelligence, artificial life and the philosophy of mind. As Clark's subtitle suggests, it proposes a close theoretical relation between brain, body and world, and implies an account of mind intriguingly different from the disembodied, individualistic picture inherited from Descartes.

This approach highlights the role of the agent's actions (and interests) in exploring its environment, and the ways in which bodily adaptations are geared specifically to interaction with that environment. It also emphasizes what workers in classical artificial intelligence have often remarked, but rarely taken to heart: that the external world can function as a rich source of information, so lessening the need for detailed on-board memory. This point was made (for instance) by Allen Newell and Herb Simon in their classic work on the psychology and artificial-intelligence modelling of cryptarithmic. Gibsonian ecological psy-

chology has long stressed these facts, and Jean Piaget's epigenetic approach posited a developmental interplay between the active organism and its environment. These ideas are now being reinforced by research on enactive cognition (in animals and machines), by cognitive and developmental neuroscience, and by situated and evolutionary robotics.

In primate vision, for example, saccadic eye movements direct the high-resolution fovea to the points of interest within the scene; movements of the head and body provide information about relative depths; perception provides affordances for adaptive action (so a space may be seen as a pathway); and specific cerebral mechanisms are devoted to distinct types of visual information. The apparent unity and all-inclusiveness of our visual phenomenology is an illusion.

For instance, you probably believe that you are experiencing the whole of this written text simultaneously. But experiments show that you would believe this even if the page consisted of nonsense-words, provided that a fixed-length 'window' of sensible (and continuously changing) text was moving along the page in precise coordination with your eyes. By implication, there is no reason to believe in a stable, 'whole-page' visual representation in the brain, or memory, underlying the visual phenomenology.

This is just one of the book's many provocative challenges to Cartesianism, and to classical (and most connectionist) cognitive science. As a philosopher, Clark is alert to the fundamental assumptions that underlie scientific research. He gives convincing arguments, backed up by neuroscientific and psychological examples, against the extreme (but increasingly fashionable) view that cognitive science has no need to posit internal representations, and that the vocabulary of dynamical systems can explain all our cognitive achievements.

Clark admits that a dynamical approach can be surprisingly helpful (in studies of human decision-making, for instance). But he argues, on philosophical and experimental grounds, that internal representations of some sort must exist. They are, however, intriguingly different from the objective and enduring representations favoured by classical artificial intelligence: most are partial, fleeting and subject centred.

The environment includes artefacts as well as natural objects, but this distinction is less clear than it may seem; for example, pheromone trails and termites' nests are part of the natural history of the creatures who made them. And, as Wittgenstein pointed out, speaking is as much a part of our own form of life as is eating or breathing. Clark regards language and (social and physical) culture as artefacts that function

to restructure the computational tasks we face into a form better suited to the human brain; that is, a basically connectionist, pattern-recognizing system. As such, they are integral to our thinking — and to our minds. In assessing the boundaries of mind, Clark questions the common (Cartesian) assumption that the human individual is theoretically basic. Much as some social psychologists and philosophers deem it impossible to define an individual human being without reference to participation in some social group, so he argues that the mind, or self, is — in a very real sense — extended across the human (social and physical) environment.

Clark's book is an excellent introduction to this new movement in cognitive science. It is clear, wide ranging, well informed, and full of fascinating examples. And, unusually, it manages to be both eminently sensible and highly provocative. □

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In deep water

Deep Atlantic: Life, Death, and Exploration in the Abyss

by Richard Ellis

Knopf: 1996. Pp. 395. \$35/Robert Hale £25

Tony Rice

Even though the deep oceans cover well over half of the Earth's surface, they have been the subject of remarkably few books for either specialist or general readerships. Only a dozen or so half-decent books have appeared in English since Wyville Thomson's *The Depths of the Sea* was first published in 1872, and most of these were written for the cognoscenti. And how many memorable science-fiction books based on the deep ocean can you think of apart from *Twenty Thousand Leagues Under the Sea* and *The Kraken Wakes*? Does this matter? Well yes, I think it does.

The vast amount of rubbish written and spoken during the recent Brent Spar debacle graphically highlighted the need to educate and inform the public and politicians about this largest of all environments on the planet, if only to try to ensure that far-reaching decisions are based on fact and not ignorance. Richard Ellis, a respected American marine artist but not a professional oceanographer, has tried to fill this gap. Although the book deals specifically with the deep Atlantic Ocean, much of it is relevant to other oceans or draws on knowledge of them.

The first part, about exploration, reviews the history of Atlantic marine science and includes sections on the Gulf Stream, and

informative accounts of the discovery and significance of seafloor spreading and hydrothermal vents. The second part, roughly two-thirds of the book, is devoted to deep-sea biology. But of some 200 pages in this section, no fewer than 150 deal with just the cephalopods, fishes and whales.

The book is generally well written, with a wealth of anecdotal detail and nice touches, such as a layman's guide to zoological nomenclature and graphic 'translations' of the unwieldy scientific Latin names that are often the only ones the unfamiliar deep-sea creatures have. As one would expect from Ellis, it is profusely illustrated by his own often beautiful drawings, particularly of the fishes, though sadly only in black and white.

Unfortunately, however, some aspects of the book detract seriously from its overall value. First, it contains an alarming number of factual errors, ranging from a picture of a *Challenger* beam trawl labelled as a plankton net to some classic taxonomic howlers. Although not particularly important, they suggest a certain lack of care. A quick read by one of the many scientists whom Ellis clearly consulted should have removed most of them.

But a much more serious shortcoming is the curious imbalance in the treatment of the major subject areas. A reader might come away with a good knowledge of the amazing morphological range of Atlantic deep-sea fishes and their almost unbelievable feeding and reproductive arrangements, yet have no idea of how the ocean actually works.

There is, for instance, almost no reference to the myriad of tiny animals inhabiting the deep sea floor, which are much more important in the overall economy of the abyss than the squids, fishes and whales put together. Nor is there any attempt to discuss the apparent enormous species diversity among these little beasts, or the relatively recently discovered small-scale variability of the deep sea that may well explain it.

I also admit to being somewhat miffed that the knowledge we have had for 15 years of the coupling between events in the surface layers and large areas of the deep sea floor, through the previously unsuspected rapid deposition of aggregated phytodetritus, is ignored. Because my research team was deeply involved in this discovery I am perhaps a little oversensitive, but by any standards it was an important discovery — and it was made in the Atlantic! It should certainly be writ large in any up-to-date book on the deep ocean.

In summary, the world needs a good, general, wide-ranging book about the deep oceans for the interested layman. This isn't it. □

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Expression cloning of GABA_B receptors uncovers similarity to metabotropic glutamate receptors

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GABA (γ -amino-butyric acid), the principal inhibitory neurotransmitter in the brain, signals through ionotropic (GABA_A/GABA_C) and metabotropic (GABA_B) receptor systems. Here we report the cloning of GABA_B receptors. Photoaffinity labelling experiments suggest that the cloned receptors correspond to two highly conserved GABA_B receptor forms present in the vertebrate nervous system. The cloned receptors negatively couple to adenylyl cyclase and show sequence similarity to the metabotropic receptors for the excitatory neurotransmitter L-glutamate.

The majority of synapses in the central nervous system (CNS) use either GABA or L-glutamate as neurotransmitters to control neuronal inhibition and excitation. GABA, like other neurotransmitters including L-glutamate, serotonin and acetylcholine, activates both ionotropic and metabotropic receptors¹⁻⁸. The ionotropic receptors are ligand-gated ion channels that convey fast synaptic transmission. In contrast, metabotropic receptors couple to G proteins (guanine-nucleotide-binding proteins) and modulate synaptic transmission through intracellular effector systems. Molecular cloning has revealed that the ionotropic receptors for L-glutamate and GABA belong to two separate gene families^{2,5}. The metabotropic receptors for L-glutamate (mGluRs) differ structurally from other 7TM G-protein-coupled neurotransmitter receptors⁹ and constitute a new gene family^{7,8}. The molecular structure of metabotropic GABA_B receptors, first reported in 1981¹⁰, has remained elusive.

GABA_B receptors modulate synaptic transmission by presynaptic inhibition of transmitter release or by increasing a K⁺-conductance responsible for long-lasting inhibitory postsynaptic potentials (late IPSPs)^{1,3,4,6,11}. Presynaptic and postsynaptic GABA_B receptor subtypes were proposed¹²⁻¹⁶. Presynaptically, GABA_B autoreceptors have been described controlling the release of GABA, whereas GABA_B heteroreceptors regulate the release of L-glutamate, noradrenaline, dopamine, 5-hydroxytryptamine, substance P, cholecystokinin or somatostatin. The induction of long-term potentiation, an associative increase in synaptic strength that may underlie the formation of some types of learning and memory, is affected by the activation of pre- and postsynaptic GABA_B receptors^{17,18}. Baclofen (Lioresal), a GABA_B receptor agonist introduced onto the market in 1972, is used to treat spasticity following multiple sclerosis and spinal injury¹⁶. Since then, other potential clinical applications, including absence epilepsy, anxiety, depression and cognition deficits, have become apparent^{3,4}. Given the physiological and clinical importance, many attempts to characterize GABA_B receptors at the molecular level have been made, but as yet have met with only limited success. The purification of a putative GABA_B receptor protein of relative molecular mass 80,000 (M_r 80K) was reported¹⁹, but no amino-acid sequence was disclosed.

With the aim of isolating a GABA_B receptor complementary DNA using expression cloning, we designed a new high-affinity GABA_B receptor antagonist. This ligand allowed the identification of cDNAs encoding two GABA_B receptor proteins, designated GABA_BR1a and -b. Amino-acid sequence analysis of the cloned receptors revealed that the metabotropic receptors for GABA and L-glutamate com-

prise a gene family. Using a [¹²⁵I]-labelled photoaffinity derivative of the new antagonist, we detected two GABA_B receptor proteins of 130K and 100K in the CNS. These GABA_B proteins are expressed in species as different as man and fish, indicating conservative evolution among vertebrates. The cloned GABA_BR1a receptor negatively couples to adenylyl cyclase when stably expressed in HEK293 cells. Recombinantly expressed GABA_BR1a and -b proteins have similar M_r s and pharmacology to native receptors. Their transcripts are abundant and expressed in all major brain structures. Taken together, this suggests that the cloned receptors correspond to the two GABA_B receptor forms that can be photoaffinity labelled in the CNS.

GABA_B receptor-specific radioligands

The agonist affinity, but not the antagonist affinity, depends on the G-protein coupling status of GABA_B receptors²⁰. For expression cloning of GABA_B receptors using a radioligand binding assay we therefore aimed at developing a high-affinity antagonist. With [¹²⁵I]CGP64213, we introduce a ligand with the highest affinity so far described ($K_d = 1.2 \pm 0.2$ nM) for the GABA_B binding site (Fig. 1). Based on the same structure we designed a photoaffinity ligand, [¹²⁵I]CGP71872 ($K_d = 1.0 \pm 0.2$ nM), which can be crosslinked to the receptor protein (Fig. 1). Both radioligands demonstrate antagonist activity at pre- as well as postsynaptic GABA_B receptors, as shown by complete suppression of L-baclofen-induced responses in electrophysiological recordings from rat CA1 hippocampal slices (M. F. Pozza, personal communication). In competition binding experiments using rat cortical cell membranes the radioligands exhibit high selectivity for GABA_B receptors (Fig. 2b and see Fig. 6). Neither L-glutamate nor compounds selective for ionotropic GABA_A⁵ or GABA_C²¹ receptors or the GABA uptake system displaced [¹²⁵I]CGP64213 or [¹²⁵I]CGP71872 from cortical GABA_B receptors (Fig. 2b and data not shown). CGP64213 or CGP71872 have no effect in binding assays for kainate, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) (agonist and co-agonist sites), muscarinic, α_1 -/ α_2 -adrenergic, β -adrenergic, 5-HT₁, 5-HT₂, 5-HT₃, histamine₁, histamine₂, adenosine₁, μ -opiate and substance P receptors at concentrations of up to 1 μ M (data not shown).

Labelling of native GABA_B receptors

[¹²⁵I]CGP71872 photoaffinity labelling of cortex, cerebellum and spinal cord cell membranes reveals two putative GABA_B proteins of 130K and 100K (Fig. 2a). These data are in contrast to previous

cis-aminocrotonic acid (CACA) or the inhibitor of the GABA uptake system, SKF89976A²², compete significantly for radioligand binding. In contrast, the GABA_B receptor agonists GABA, 3-aminopropylphosphinic acid (APPA) and L-baclofen compete with [¹²⁵I]CGP71872 for binding. As another known criterion²³, L-baclofen competes more potently than D-baclofen. The GABA_B receptor antagonists CGP54626A²⁴, CGP35348²⁴ and the non-radioactive photoaffinity ligand are also effective displacers of [¹²⁵I]CGP71872 at native receptors. For all ligands tested, there is no visible difference in the displacement of [¹²⁵I]CGP71872 at the 130K and 100K proteins, indicating a qualitatively similar binding pharmacology for the two receptors.

Expression cloning of a GABA_B receptor cDNA

As with many neurotransmitter receptors, biochemical isolation of GABA_B receptors was hindered by the lack of (1) ligands that bind under solubilizing conditions with high affinity to the protein and (2) cell lines that express significant amounts of receptor protein. We therefore explored cloning strategies using *Xenopus* oocytes and electrophysiological measurements, without success. To overcome these problems, we used an expression cloning procedure in which we screened for [¹²⁵I]CGP64213 binding at transfected COS-1 cells.

A high density of GABA_B receptor binding sites is found in cortex and cerebellum of 7-day-old rats²⁵. Those tissues were therefore used as a source to construct a cDNA library (complexity 2×10^6 clones) in the expression vector pcDNA1. The library was divided into pools of 2,000 cDNA clones each and individual pools were transfected into COS-1 cells and screened for radioligand binding. A total of 310 pools were analysed until one positive pool and, after serial subdivisions, a single clone containing a 4.4 kilobase (kb) cDNA insert was identified. This cDNA clone encodes a protein of 960 amino acids, designated GABA_BR1a (Fig. 3a). When expressed in COS-1 cells the GABA_BR1a protein has an apparent M_r that is similar to that of the 130K GABA_B receptor present in brain, as shown by photoaffinity labelling (Fig. 3c). The amino-terminal 16 amino acids of GABA_BR1a probably constitute the signal peptide²⁶. The calculated M_r of the mature protein is 106K, similar to the M_r of the native 130K GABA_B receptor protein after treatment with *N*-glycosidase F (Fig. 2c). Consistent with *N*-glycosylation of native GABA_B receptors, GABA_BR1a contains several consensus *N*-glycosylation sites in the N-terminal extracellular domain (Fig. 3a). Putative substrate sites²⁷ for casein kinase II and protein kinase C are found in cytoplasmic domains, suggesting that receptor activity could be regulated by phosphorylation.

We screened the cDNA library by low-stringency hybridization using the GABA_BR1a cDNA as a probe. Several clones distinct from the GABA_BR1a cDNA were isolated. A 2.9 kb cDNA encodes a protein of 844 amino acids, designated GABA_BR1b (Fig. 3a). The mature GABA_BR1b differs from GABA_BR1a in that the N-terminal 147 residues are replaced by 18 different residues (Fig. 3a). Presumably, the GABA_BR1a and -b receptor variants are derived from the same gene by alternative splicing. Transient expression of the GABA_BR1b cDNA in COS-1 cells yields a protein with a similar M_r to the 100K receptor detected in brain tissue (Fig. 3c). The calculated M_r of the mature GABA_BR1b is 92K, similar to that of

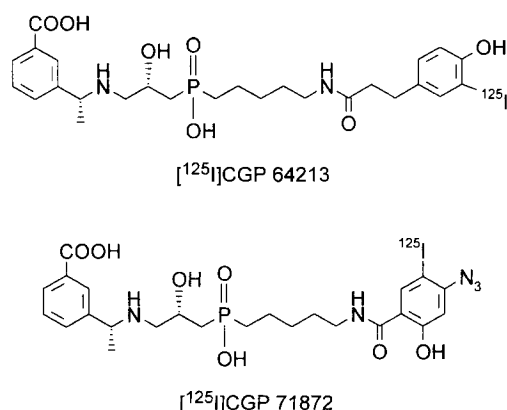


Figure 1 Chemical structures of the GABA_B receptor-specific ligands [¹²⁵I]CGP64213 and [¹²⁵I]CGP71872.

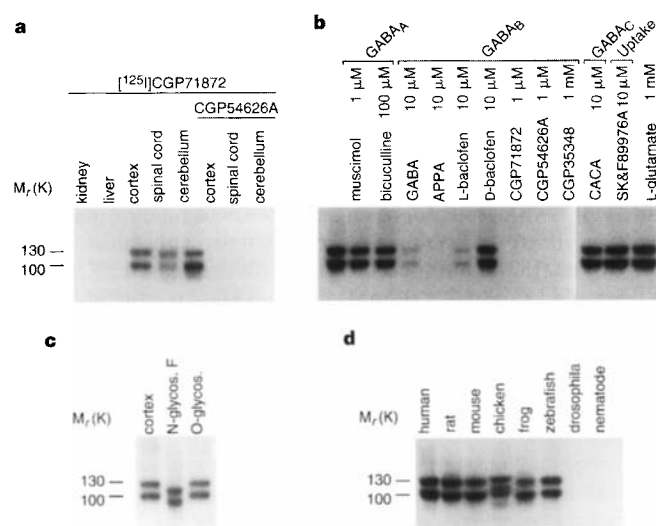


Figure 2 Photoaffinity crosslinking of GABA_B receptor proteins. Cell membranes of the tissues indicated were photoaffinity labelled with [¹²⁵I]CGP71872 and subjected to SDS-PAGE and autoradiography. **a, b**, Selectivity of the photoaffinity ligand [¹²⁵I]CGP71872. **a**, Differential distribution of GABA_B receptor variants of 130K and 100K in tissues of the nervous system. [¹²⁵I]CGP71872 binding is inhibited by addition of 1 μM of CGP54626A, a selective GABA_B receptor antagonist²⁴. **b**, Competition of [¹²⁵I]CGP71872 labelling by different ligands. Incubation of membrane extracts with the photoaffinity ligand was carried out in the presence of competitor substances at the concentrations indicated. **c**, GABA_B receptors are *N*-glycosylated. Photoaffinity-labelled rat cortex cell membranes were incubated with 0.4 units *N*-glycosidase F or 0.6 milliunits *O*-glycosidase (Boehringer Mannheim). **d**, Photolabelling of GABA_B receptors from different species. Brain tissues from the species indicated were labelled as described in Methods. In the case of *Drosophila melanogaster* and *Haemochus concolor* whole animals were analysed.

the deglycosylated 100K brain receptor (Fig. 2c). Northern blot analysis (Fig. 4) indicates that transcripts for both GABA_BR1a and -b are abundant in brain and neither represent cloning artefacts nor rare aberrant splice events. In support of this, we found (1) several full-length GABA_BR1a and -b clones in rat brain and spinal cord cDNA libraries and (2) several partial human GABA_BR1a and -b cDNA clones (data not shown).

GABA_B receptors and mGluRs

The GABA_BR1a/b proteins are considerably larger than classic G-protein-coupled receptors⁹ and are similar in size to mGluRs^{7,8} ranging from 872 to 1,203 amino-acid residues. Indeed, Blast²⁸ and FASTA²⁹ database searches indicate that GABA_BR1 is most closely related to members of the mGluR gene family which contains eight subtypes as well as a Ca²⁺-sensing receptor³⁰. Bestfit²⁷ sequence alignments of GABA_BR1a with these receptors reveals 18–23% amino-acid sequence identity and 43–48% related residues (Fig. 3a and data not shown). The similarity between GABA_BR1a and the mGluRs is in the same range as for distant subunits of the ionotropic glutamate receptor gene family². Although the amino-acid sequence identity between GABA_BR1a and the mGluRs is low, the conservation of the structural architecture is clearly evident from the hydrophobicity profiles³¹ (Fig. 3b). As for the mGluRs, a large N-terminal extracellular domain precedes seven closely spaced putative transmembrane domains, indicative of G-protein-coupled receptors. The sequence similarity of GABA_BR1 to individual mGluRs is not confined to specific domains; notably it is not restricted to the transmembrane regions (Fig. 3a). A total of 208 amino-acid residues are conserved in all mGluRs and 27 of these are preserved in GABA_BR1 (Fig. 3a; empty circles), further emphasizing that GABA_B receptors and mGluRs are related. Twenty-one cysteine residues are conserved in all mGluRs (Fig. 3a; filled circles) and are considered a hallmark of the mGluR gene family. Most cysteine residues are not conserved in GABA_BR1. Nine cysteines are closely spaced in the N-terminal extracellular domain of mGluRs, a part of the protein referred to as the cysteine-rich region. Such a region is missing in GABA_BR1. Blast and FASTA database searches with the GABA_BR1 sequence also reveal weak similarities to guanylyl cyclases, that is, the natriuretic peptide receptor³², to selectins³³ and to the complement receptor type I³⁴, as well as to bacterial amino-acid-binding proteins^{35,36}. No significant sequence similarity is found to GABA_A or GABA_C receptors nor to G-protein-coupled

receptors⁹ other than the mGluRs.

In their N-terminal extracellular domain, mGluRs contain two lobes with structural similarity to the amino-acid binding sites of bacterial proteins³⁷. It has been proposed that these lobes constitute the L-glutamate binding site of mGluRs. A FASTA search reveals that a structural similarity to bacterial amino-acid binding proteins is also evident for the N-terminal extracellular domain of GABA_BR1 (Fig. 3a; arrows). Strikingly, the N-terminal extracellular domain of the smaller GABA_BR1b receptor is limited to the region with structural similarity to the bacterial proteins.

It was shown for mGluRs that the second intracellular loop (Fig. 3a; ICL2) determines the specificity of G-protein coupling³⁸. In this region, GABA_BR1 does not show any significant sequence similarity to mGluRs. Therefore one of several possible signal transduction pathways, as described for mGluRs^{7,8}, cannot be inferred. However, as in the mGluRs, the ICL1 and ICL3 are small and the ICL2 region is rich in basic residues and therefore is expected to be involved in G-protein interaction.

Spatial distribution of GABA_BR1 transcripts

The tissue distribution of GABA_BR1a and -b mRNA was examined by northern blot analysis (Fig. 4) and *in situ* hybridization (Fig. 5). Northern blots hybridized with a probe containing sequences common to both GABA_BR1a and -b (Fig. 4a; pan probe) revealed RNAs of 4.3 to 4.4 kb and 3 kb present in brain and testis, but not in the other tissues analysed (Fig. 4b). The size of these transcripts indicates that the cloned 4.4 kb GABA_BR1a cDNA represents a nearly full-size mRNA. Separate analysis of GABA_BR1a and -b transcripts (Fig. 4a; probes R1a and R1b) revealed RNAs of 4.4 kb and ~1.8 kb for R1a and RNAs of 4.3 kb and 3 kb for R1b, clearly demonstrating the expression of both receptor variants in brain (Fig. 4b). The sequences of the 3 kb and 1.8 kb transcripts are unknown, but the 1.8 kb RNAs appear too short to encode a functional GABA_B receptor.

In situ hybridization studies were carried out using the pan probe that does not hybridize to the 1.8 kb RNAs (Fig. 5). GABA_BR1 receptor transcripts are abundant in all cerebral cortical layers, in the pyramidal cell layers of the hippocampus, the granular cell layer of the dentate gyrus (Fig. 5a, b) and in the basal ganglia (data not shown), including the caudate putamen, nucleus accumbens and olfactory tubercle. In the cerebellum, transcripts are found in abundance in the Purkinje cells and at moderate levels in the

Table 1 Binding affinities of native and recombinant GABA_B receptors

	Rat cerebral cortex		GABA _B R1a		GABA _B R1b	
Agonists	IC ₅₀ (μM)	Hill coeff. <i>n</i> _H	IC ₅₀ (μM)	Hill coeff. <i>n</i> _H	IC ₅₀ (μM)	Hill coeff. <i>n</i> _H
GABA	0.14 ± 0.03	0.55 ± 0.03	22.9 ± 2.4	0.84 ± 0.04	31.1 ± 4.0	0.89 ± 0.06
APPA	0.018 ± 0.001	0.57 ± 0.02	2.3 ± 0.5	0.75 ± 0.06	2.8 ± 0.2	0.81 ± 0.06
L-baclofen	0.21 ± 0.05	0.54 ± 0.04	25.1 ± 7.8	0.74 ± 0.14	35.1 ± 4.2	0.80 ± 0.02
CGP47656	0.29 ± 0.08	0.74 ± 0.04	10.8 ± 2.2	0.73 ± 0.11	15.0 ± 2.4	0.79 ± 0.10
Antagonists						
CGP56999A	0.0004 ± 0.0001	1.17 ± 0.06	0.0007 ± 0.0002	1.14 ± 0.13	0.0008 ± 0.0001	0.99 ± 0.05
CGP62349	0.0007 ± 0.0001	1.07 ± 0.06	0.0011 ± 0.0002	0.87 ± 0.03	0.0016 ± 0.0008	0.88 ± 0.05
CGP64213	0.0016 ± 0.0003	1.08 ± 0.02	0.0023 ± 0.0001	1.01 ± 0.05	0.0031 ± 0.0001	1.00 ± 0.04
CGP54626A	0.0022 ± 0.0005	0.99 ± 0.02	0.0015 ± 0.0001	0.88 ± 0.03	0.0020 ± 0.0001	1.01 ± 0.05
CGP71872	0.0024 ± 0.0008	1.08 ± 0.03	0.0036 ± 0.0004	1.05 ± 0.18	0.0051 ± 0.0004	1.02 ± 0.07
CGP35348	4.2 ± 0.8	0.85 ± 0.02	17.2 ± 3.1	0.67 ± 0.06	21.7 ± 1.3	0.81 ± 0.09
2-OH-saclofen	12.4 ± 1.1	0.84 ± 0.04	82.7 ± 10.5	0.85 ± 0.06	87.5 ± 7.9	0.74 ± 0.04
Saclofen	28.3 ± 1.9	0.81 ± 0.04	290.0 ± 35.3	0.73 ± 0.04	366.0 ± 32.2	0.65 ± 0.10
SCH50911	0.27 ± 0.02	0.96 ± 0.02	0.38 ± 0.03	0.87 ± 0.01	0.43 ± 0.04	0.78 ± 0.04

Inhibition of [¹²⁵I]CGP64213 binding to native and recombinant GABA_B receptors by GABA_B agonists and antagonists. Rat cerebral cortex membranes and membranes from COS-1 cells transiently transfected with the GABA_BR1a and -b cDNAs were used. IC₅₀ values and Hill coefficients were fitted using nonlinear regression (PRISM program, Graph Pad Software Inc., San Diego). Values are means ± s.e.m. of 3 independent experiments; APPA, 3-aminopropylphosphinic acid.

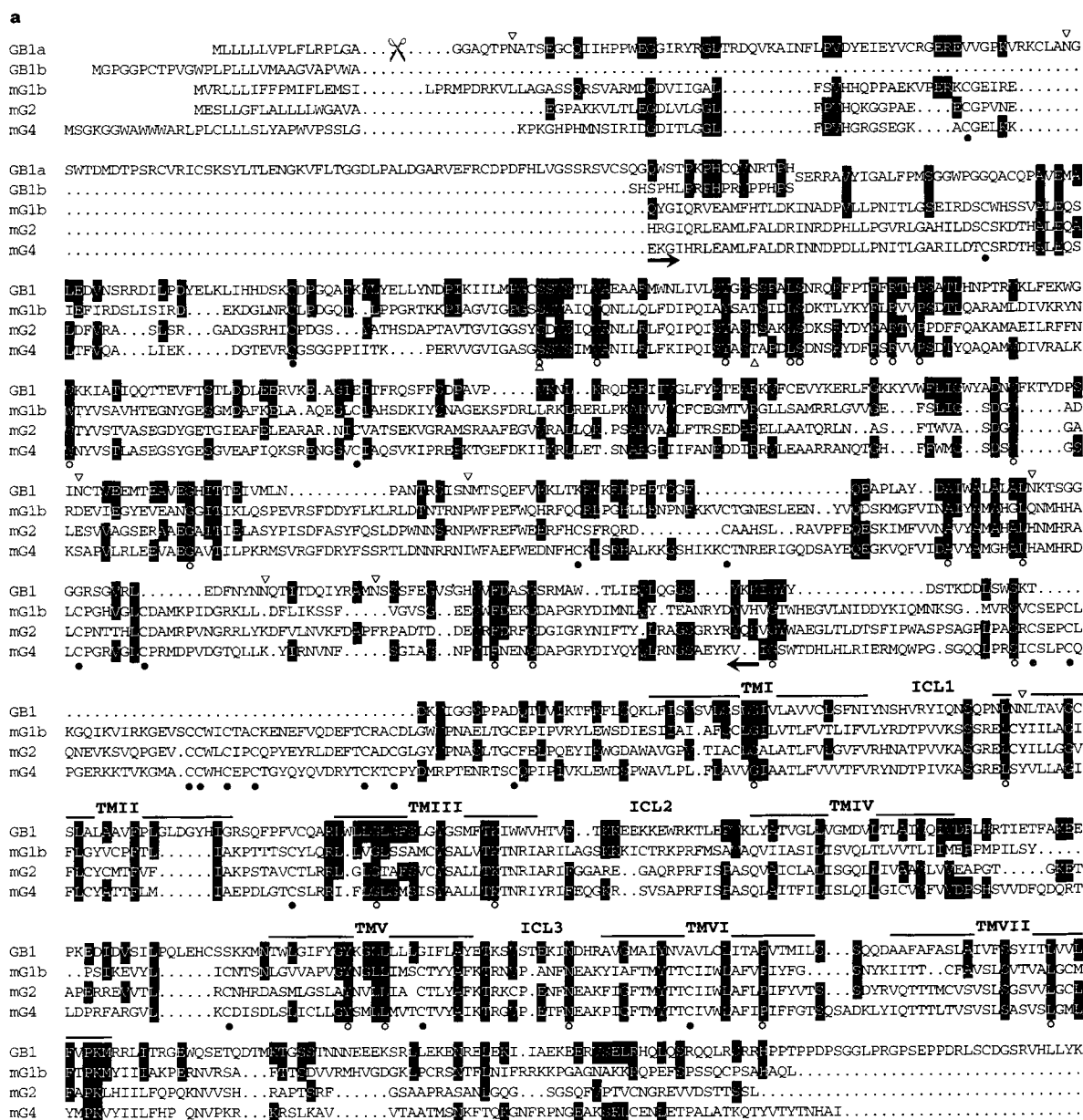
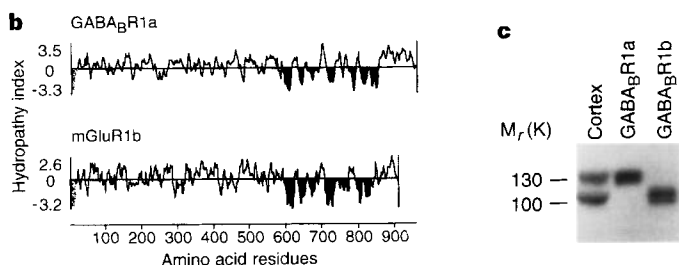


Figure 3 a. Alignment of the rat GABA_BR1 (GB1a), GABA_BR1b (GB1b), mGluR1b (mG1b), mGluR2 (mG2) and mGluR4 (mG4) amino-acid sequences. The sequences of GABA_BR1a and GABA_BR1b differ at the N terminus and are otherwise identical. Amino acids that are identical in the N-terminal sequences of GABA_BR1a and GABA_BR1b and amino acids that are conserved among GABA_BR1 and one or more of the three mGluRs are boxed in black. The putative transmembrane domains (TMI-TMVI) and intracellular loops (ICL1-ICL3) are indicated. Proposed signal peptide cleavage sites²⁶ are marked with scissors. Putative *N*-glycosylation sites are found at amino-acid positions 7, 67, 392, 423, 465, 485, 497 and 614 of the predicted mature GB1a and are marked by inverted triangles. Residues conserved throughout the mGluRs and GABA_BR1 are indicated by empty circles, conserved cysteines of mGluRs are denoted by filled circles. Residues in mGluR1³⁷ affecting L-glutamate affinity are indicated by triangles, arrows delimit the region where GABA_BR1 has structural similarity with bacterial amino-acid binding proteins. **b.** Hydropathy profiles of the GABA_BR1 and mGluR1b sequences, computed according to Kyte and Doolittle³¹ using sequence analysis programs from the University of Wisconsin Genetics Computer Group²⁷. Black colouring indicates the positions of the hydrophobic



transmembrane domains, grey colouring the N-terminal signal peptides. **c.** [¹²⁵I]CGP71872 photoaffinity labelling of cell membranes from rat cortex and COS-1 cells transiently transfected with the GABA_BR1a and GABA_BR1b cDNA. Autoradiography of a SDS-PAGE (6%) is shown. Recombinant GABA_B receptor proteins sometimes appear as a doublet band in SDS-PAGE, probably due to photoaffinity labelling of incompletely processed receptors.

granular layer (Fig. 5d, e). GABA_BR1 mRNAs are expressed in neuronal but not glial cells (for example, Fig. 5c). A direct comparison of the distribution of transcripts and protein in the CNS is difficult. There is a close overlap of GABA_BR1 mRNA and GABA_B receptor binding sites, as assessed by autoradiography using radioligands^{39,40}, in the medial habenula (Fig. 5a), the medial geniculate nucleus (Fig. 5b) and the interpeduncular nucleus. In other CNS areas the regional abundance of transcripts and binding sites differs. In the cerebral cortex transcript levels are higher in layer VIb than in layers II–V, whereas the opposite has been reported for the density of receptor binding sites^{39,40}. In the hippocampal formation, expression of the mRNA is detected in the pyramidal and granule cell layers, whereas receptor binding sites were reported in the molecular layers. In the cerebellum, transcripts are found in the Purkinje cells and in the granular layer (Fig. 5d, e), whereas

GABA_B receptor binding sites were reported to be most abundant in the molecular layer, but also to be present in the granular layer^{39,40}. In the cerebellum GABA_B receptors may therefore be located on Purkinje cell dendrites and granule cell parallel fibres, in agreement with previous studies^{39–41}.

Pharmacological profiles

To compare the pharmacological profiles of GABA_BR1a/-b expressed in mammalian cells (COS-1/HEK293) and GABA_B receptors present in rat cerebral cortex, we analysed the inhibition of [¹²⁵I]CGP64213 binding by selected GABA_B receptor antagonists and agonists (Fig. 6a, b; Table 1). The inhibition curves for the potent GABA_B receptor antagonists CGP56999A²⁴ and CGP54626A²⁴ at native and recombinant receptors are almost identical (Fig. 6a). For all potent antagonists tested the 50%

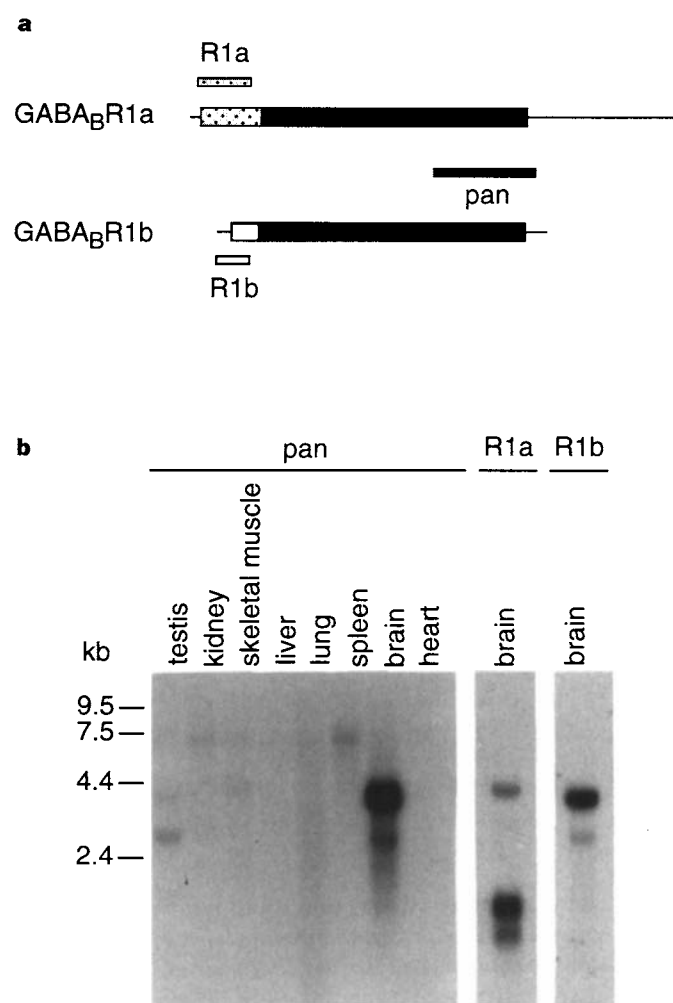


Figure 4 Northern blot analysis of GABA_BR1a and -b mRNA expression. **a**, Schematic diagram showing the localization of the probes used. Coding sequences are boxed, untranslated sequences are indicated by a line. Only N-terminal sequences of GABA_BR1a and -b differ. The R1a probe corresponds to nucleotides 1 to 405 of the GABA_BR1a cDNA, the R1b probe to nucleotides 16 to 259 of the GABA_BR1b cDNA. The pan probe is common to both GABA_BR1a and -b and is derived from nucleotides 2,462 to 3,234 of the GABA_BR1a cDNA. **b**, Rat multiple tissue northern blots (Clontech, Palo Alto) with 2 µg poly(A)⁺ RNA per lane were hybridized to random-primed ³²P-labelled probes.

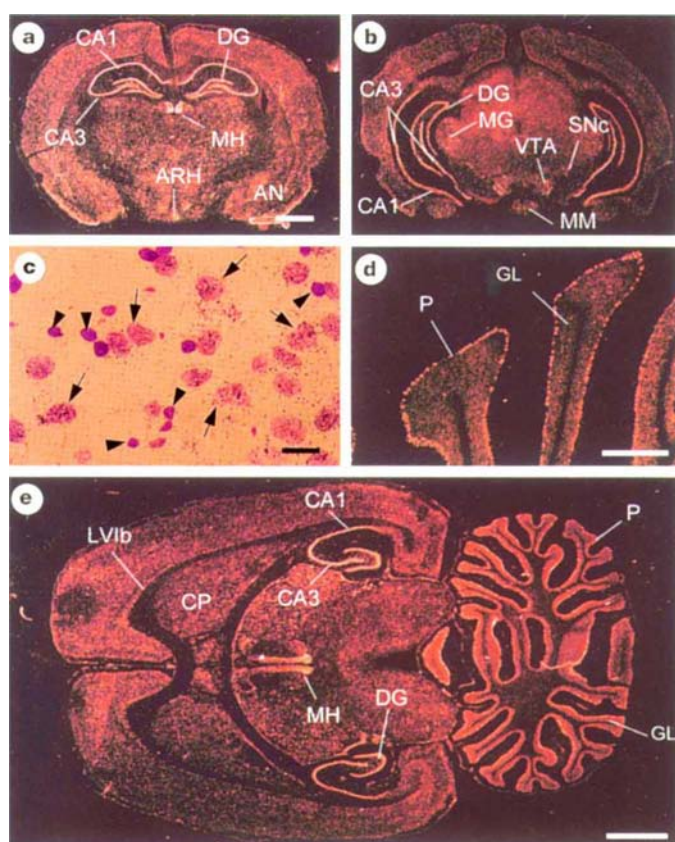
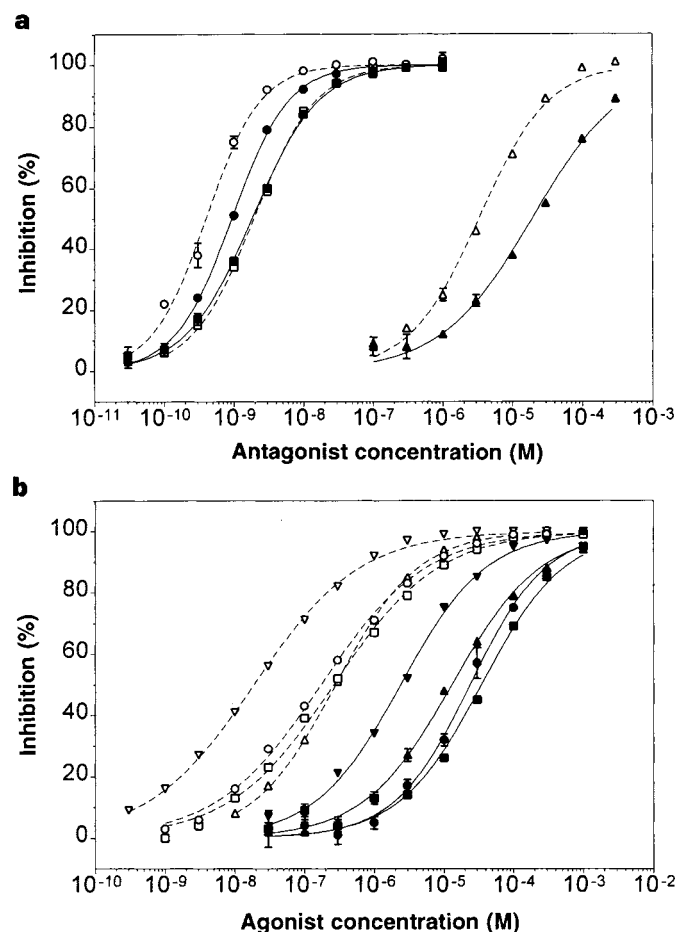


Figure 5 *In situ* hybridization analysis of GABA_BR1 transcripts in rat brain. Tissue sections were hybridized to ³⁵S-labelled antisense probes. Darkfield illuminations of representative autoradiograms of coronal (**a**, **b**) and horizontal sections (**c**, **d**), and a brightfield illumination (**e**) are shown. **a**, Dorsal hippocampus plane; **b**, ventral hippocampus plane; **c**, CA3 field of the hippocampus; **d**, lobules of the cerebellar cortex; **e**, dorsal tier of the brain. Transcripts are abundant in all cerebral cortical layers, especially in the layer VIb (LVib), in the pyramidal cell layer of the CA1–CA3 subfields of the hippocampus as well as in the granular layer of the dentate gyrus (DG) and in the medial habenula (MH). GABA_BR1 mRNA is detected in the medial geniculate nucleus (MG), in the substantia nigra, pars compacta (SNc), in the ventral tegmental area (VTA) and in several thalamic, amygdaloid (AN) and hypothalamic nuclei, such as the arcuate nucleus of the hypothalamus (ARH) and mammillary bodies of the hypothalamus (MM). In the cerebellum, high levels of transcripts are found in the Purkinje cells (P) and moderate levels in the granular layer (GL). Arrows indicate neuronal, arrowheads glial cells. The sections were exposed to nuclear emulsion for 12 days. No hybridization signal was observed with radiolabelled sense probes. Scale bars, 2 mm (**a**, **b**), 10 µm (**c**), 30 µm (**d**), 1.3 mm (**e**).



inhibitory concentration (IC₅₀) values are similar for native and cloned GABA_B receptors (Table 1). The Hill coefficients are close to 1, indicating single high-affinity binding sites. Low-affinity GABA_B receptor antagonists, such as CGP35348²⁴, saclofen⁴ and 2-OH-saclofen⁴, have 4- to 13-fold reduced affinities at recombinant as compared with native receptors (Fig. 6a; Table 1). For the GABA_B receptor agonists GABA, APPA and L-baclofen, the rank order of affinities is identical at native and recombinant receptors (Fig. 6b). However, the affinities for these agonists at the recombinant receptors are reduced by 100-fold (GABA_BR1a) to 150-fold (GABA_BR1b) when compared to their affinities at rat cortex receptors (Table 1). In the case of the partial agonist CGP47656⁴² a 30- and 50-fold lower affinity is found. The antagonists saclofen and 2-OH-saclofen may act as weak partial agonists⁴³ and therefore, similar to the agonists tested, show a difference in the ligand-displacement IC₅₀ values at recombinant versus native receptors (Table 1). The Hill coefficients for full agonists are between 0.5 and 0.6 for native receptors and 0.7 and 0.9 for recombinant receptors (Table 1), suggesting multiple affinity states. The discrepancy in agonist binding affinity between native and recombinant receptors could be due to the presence of additional, pharmacologically distinct, GABA_B receptor subtypes in the brain. However, alternative explanations are possible and are discussed below.

Based on the effectiveness of some ligands in preferentially modulating the release of one versus another neurotransmitter, the existence of auto- and heteroreceptor subtypes has been

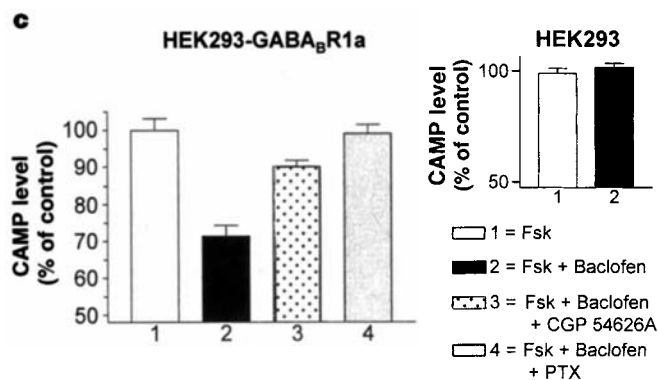


Figure 6 Pharmacological properties of native and recombinant GABA_B receptors. **a**, Inhibition of [¹²⁵I]CGP64213 binding to rat cerebral cortex GABA_B receptors (empty symbols) and to GABA_BR1a stably expressed in HEK293 cells (filled symbols) by the GABA_B receptor antagonists CGP56999A (●), CGP54626A (■) and CGP35348 (▲). **b**, Inhibition of [¹²⁵I]CGP64213 binding to GABA_B receptors in cell membranes from rat cerebral cortex (empty symbols) and from HEK293 cells stably expressing GABA_BR1a (filled symbols) by the GABA_B receptor agonists GABA (●), L-baclofen (■), APPA (▼) and the partial agonist CGP47656 (▲). Results from typical experiments performed in triplicate are shown. Bars indicate standard errors of the mean (s.e.m.). The curves were fitted using nonlinear regression (Graph Pad PRISM program, Graph Pad Software Inc., San Diego, USA). The dissociation constants *K_d* of the binding of [¹²⁵I]CGP64213 were 2.1 ± 0.2 nM for GABA_BR1a and 1.2 ± 0.2 nM for rat brain GABA_B receptors. The maximal numbers of [¹²⁵I]CGP64213 binding sites (*B_{max}*) at recombinant (GABA_BR1a) and native receptors were 43.0 ± 1.1 and 3.7 ± 0.9 pmol mg⁻¹ protein (*n* = 3), respectively. **c**, GABA_BR1a mediates inhibition of adenylyl cyclase. HEK293 cells stably expressing GABA_BR1a were treated with 20 μM forskolin (Fsk) to stimulate cAMP formation (100%). Fsk induced cAMP accumulation is reduced significantly (*P* < 0.001; Dunnett's *t*-test) upon simultaneous addition of 300 μM L-baclofen. The effect of L-baclofen is antagonized in the presence of 10 μM CGP54626A. Preincubation of the cells with 10 ng ml⁻¹ pertussis toxin (PTX) for 15–20 h completely abolishes the effect of L-baclofen. No L-baclofen response is observed in non-transfected HEK293 cells (insert). Bars represent mean values + s.e.m. of at least three independent experiments performed in quadruplicate.

proposed^{12–16,44}. The binding affinities of CGP35348 (Fig. 6a), CGP36742 and CGP52432 (data not shown), compounds reported to exhibit some subtype selectivity, were measured at GABA_BR1a and -b receptors. The -b variant has slightly lower affinities for all agonists and antagonists tested, but otherwise no pharmacological difference was observed. This implies that only sequences common to both GABA_BR1a and -b are directly involved in ligand binding.

GABA_B receptors negatively couple to adenylyl cyclase

GABA_B receptors are described to inhibit adenylyl cyclase activity, stimulate phospholipase A₂, activate K⁺-channels, inactivate voltage-dependent Ca²⁺-channels and modulate inositol phospholipid hydrolysis^{41,45,46}. As GABA_BR1a and -b have identical sequences in all domains predicted to be intracellular they are expected to be able to couple to the same effector systems. Using rat cortical slice preparations, L-baclofen was found to reduce forskolin-stimulated cAMP accumulation by about 40%⁴⁵. We analysed the ability of GABA_BR1a stably expressed in HEK293 cells to reduce forskolin-stimulated cAMP accumulation (Fig. 6c). We chose concentrations of forskolin and L-baclofen that should produce a maximal effect. Forskolin stimulates cAMP levels in HEK293 cells to more than 10 times the basal level. Stimulation of recombinantly expressed GABA_B receptors by co-addition of 300 μM L-baclofen reduces forskolin-stimulated cAMP accumulation by about 30%. This inhibition is antagonized by CGP54626A, a GABA_B receptor antagonist. The modulation of adenylyl cyclase activity by

GABA_BR1a is sensitive to pertussis toxin, indicating that in HEK293 cells, which are deficient in G_o (ref. 47), GABA_BR1 couples to G_i. As a control, L-baclofen does not inhibit forskolin-stimulated cAMP formation in untransfected HEK293 cells (Fig. 6c). In preliminary experiments using GABA_BR1a, we were unable to detect positive coupling to adenylyl cyclase or coupling to the phospholipase C effector system. GABA_BR1a/-b cRNA was injected into *Xenopus* oocytes, but no Ca²⁺-dependent Cl⁻ currents or K⁺ currents were detectable upon L-baclofen (100 μM) superfusion (J. Mosbacher, personal communication).

Discussion

Except for the GABA_B receptors, the receptors for the major known neurotransmitters have been cloned. We have reported here the isolation of cDNAs encoding the GABA_BR1a/-b proteins that exhibit many of the properties expected of GABA_B receptors: (1) the amino-acid sequence is highly indicative of 7TM G-protein-coupled receptors and indicates a common gene family for the metabotropic receptors for GABA and L-glutamate; (2) when expressed in HEK293 cells, the cloned receptors negatively couple to adenylyl cyclase (Fig. 6c), as described for native GABA_B receptors⁴⁵; (3) recombinant GABA_BR1a and -b proteins have similar *M_r* to GABA_B proteins present in the nervous system of several vertebrate species (Figs 2d and 3c); and (4) the rank order of the binding activities of antagonists and full agonists are identical at recombinant and rat cerebral cortex GABA_B receptors (Fig. 6a, b; Table 1). The above data suggest that the cloned receptors are the molecular correlates of the two GABA_B receptors that we can distinguish in different brain structures.

Some findings, however, require further investigation. In contrast to antagonists, agonists have significantly lower binding activities at recombinant as opposed to native receptors (Fig. 6b; Table 1). No change in binding activity is observed when GABA_BR1a and -b are coexpressed in COS-1 cells (IC₅₀ values for GABA, APPA and L-baclofen are 18 μM, 3.2 μM and 29 μM, respectively), arguing against a heteromeric protein complex with increased agonist affinity. Low agonist affinity could be inherent to the cloned receptors, but it could also reflect properties of the heterologous expression system. For example, inefficient G-protein coupling or receptor desensitization could influence agonist affinity. It has been shown for GABA_B receptors²⁰ and other G-protein-coupled receptors⁴⁸ that the binding affinity of agonist, but not of antagonists, is dependent on the G-protein coupling status. GTP, or its stable analogue Gpp(NH)p, reduces the affinity of native GABA_B receptors to agonists by uncoupling the receptors from their G proteins. We determined that at cortex GABA_B receptors the IC₅₀ values for GABA, APPA and L-baclofen are ~10-fold higher in the presence of 300 μM Gpp(NH)p (data not shown). These IC₅₀ values are still about 10-fold lower than the values obtained at recombinant receptors but indicate that G-protein coupling could at least in part account for the differences in agonist affinity observed. We did not detect any difference in agonist binding affinity between recombinant GABA_B receptors expressed in COS-1 (Table 1) and HEK293 cells (Fig. 6a, b), even though in the latter the receptor is functionally coupled (Fig. 6c). However, in HEK293 cells only a fraction of the receptors might be coupled to G proteins, which could be the reason that a putative increase in agonist affinity was not measurable. Low- and high-affinity GABA_B receptors have been described in functional assay systems⁴⁶. In retrospect, should the cloned receptors represent a low-affinity GABA_B receptor subtype, these receptors would not have been detected in previous autoradiographic studies, as only tritiated agonists were used to acquire such data^{39,40}. This may explain some of the discrepancy found between the GABA_B receptor *in situ* hybridization pattern described here (Fig. 5) and the distribution of binding sites reported earlier^{39,40}. It has often been suggested that postsynaptic GABA_B receptors are sensitive to PTX⁴. Although earlier experiments are

not readily comparable to our experiments, the PTX sensitivity of the coupling of GABA_BR1a in HEK293 cells (Fig. 6c) may indicate that this receptor could fulfil a postsynaptic role. As with the majority of neurotransmitter receptors, the cloning of additional GABA_B receptors or the identification of more splice variants may explain the pharmacological differences reported previously. However, the receptors described here may also be able to couple to more than one G protein and multiple second messenger pathways and the pharmacological differences in functional assays may depend on the types and efficacies of effector systems available^{45,49}.

The ubiquitous distribution of GABA_B receptors and their pharmacological actions suggest that they are likely targets in therapy, but their clinical importance awaits a deeper understanding of their molecular and functional diversity. The cloning of GABA_B receptor cDNAs now provides the tools to study receptor heterogeneity and to correlate the known pre- and postsynaptic effects of GABA_B receptors with distinct receptor molecules. □

Methods

Ligands. [¹²⁵I]CGP64213, the GABA_B receptor antagonist used for expression cloning, and [¹²⁵I]CGP71872, the photoaffinity ligand used to tag GABA_B receptor protein covalently, were synthesized from ethyl (1,1-diethoxyethyl) phosphinate⁴². Both ligands were labelled to a specific radioactivity of >2,000 Ci mmol⁻¹ (ANAWA AG, Wangen, Switzerland). Experimental details on the syntheses of both ligands will be reported elsewhere. All the other ligands used in this study were synthesized in-house^{3,24,42}. Their chemical structures are available as supplementary information.

Expression cloning of GABA_BR1a. Oligo (dT) primed double-stranded cDNA was synthesized from 5 μg poly(A)⁺ RNA using a commercial cDNA synthesis system (Amersham, Superscript II reverse transcriptase from Gibco BRL). After the addition of *Bst*XI adaptors (Invitrogen), cDNAs >2 kb were gel-purified and ligated into pcDNA1 (Invitrogen). Aliquots of the ligation mixture were transformed into electrocompetent MC1061/P3 *Escherichia coli* cells. Plasmid DNA was isolated from pools of 2,000 bacterial colonies obtained after the initial round of transformation. The DNA was introduced into COS-1 cells by DEAE-dextran transfection. COS-1 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM, 10% fetal calf serum, 15 μg ml⁻¹ gentamycin). The cells were washed for 15 min in PBS, incubated for 9 min in 1 mg ml⁻¹ (w/v) DEAE-dextran (Pharmacia) in PBS at room temperature, rinsed in PBS, and plasmid DNA was added (4 μg DNA in 540 μl PBS per 9 cm plate) and incubated for 30 min at 37°C. Subsequently DMEM medium containing 10% NU-serum (Collaborative Research) and 80 μM chloroquine (Sigma) was added. After 4 h incubation at 37°C the medium was removed and the cells were incubated for 2 min in 10% (vol/vol) dimethyl sulphoxide in PBS. The cells were rinsed in PBS, cell culture medium was added to the culture dishes and the cells were grown for an additional 2 to 3 days. COS-1 cells transfected with the plasmid pools were analysed for GABA_B receptor expression using [¹²⁵I]CGP64213 binding. The cells were cooled on an ice bath, washed twice with ice-cold Krebs-Tris buffer (20 mM Tris-Cl pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 4.7 mM KCl, 1.8 mM CaCl₂), and incubated for 80 min at room temperature with 0.2 nM of [¹²⁵I]CGP64213 in Krebs-Tris buffer. The cells were then cooled and washed twice with ice-cold Krebs-Tris buffer. Subsequently the dishes were air-dried and the walls of the plates were removed. Kodak X-OMAT AR films, together with intensifying screens, were exposed to the plates for 2 to 3 weeks at -80°C.

Cross-hybridization screening. Bacterial colony hybridization was done using the ³²P-labelled GABA_BR1a cDNA as probe (Boehringer Mannheim DNA labelling kit). Hybridization was carried out in 0.5 M Na₂HPO₄, pH 7.2, 7% SDS, 1 mM EDTA at 60°C and wash steps were with 0.5 × SSC, 0.1% SDS at 55°C.

In situ hybridization and northern blot analysis. *In situ* hybridization histochemistry using 10 μm coronal and horizontal cryosections (postfixed with 4% paraformaldehyde) of rat brain (male Tif RAI f [SPF] rats weighing 250 g) was as described⁵⁰. ³⁵S-UTP/³⁵S-ATP-labelled riboprobes were generated from a cDNA fragment that is common to both GABA_BR1a and -b. Post-hybridization was performed under high stringency conditions (63% forma-

mide, 80 °C for the post-hybridization wash). Slides were dipped into nuclear emulsion and exposed for 15 days. Northern blots were hybridized at 50 °C in the above solution containing 50% (vol/vol) formamide and filters washed with $0.1 \times \text{SSC}$, 0.1% SDS at 68 °C.

Ligand binding assay. Competition binding experiments were performed with COS-1, HEK293 or rat cortex cell membranes. To prepare membranes, cells were homogenized in Krebs-Tris buffer, centrifuged for 30 min at 40,000g and the pellet resuspended. Synaptic membranes were prepared as described⁵¹. Membranes were suspended in Krebs-Tris buffer at a concentration of approximately $50 \mu\text{g ml}^{-1}$ and incubated with 0.1 nM [¹²⁵I]CGP64213 for 90 min in the presence or absence of competitor ligands. The incubation was terminated by filtration through GF/C Whatman glass fibre filters.

Photoaffinity labelling. Cell membranes were incubated in the dark with 0.6 nM [¹²⁵I]CGP71872 for 1 h at room temperature. The incubation was terminated by centrifugation at 20,000g for 10 min at 4 °C. The pellet was washed in buffer to remove unbound from bound photoaffinity label, resuspended and illuminated with UV light (365 nm, 24 W) for 3 min. The suspension was centrifuged (20,000g, 20 min), the pellet washed in buffer and dissolved in SDS-PAGE loading buffer.

cAMP assay. The GABA_BR1a cDNA was cloned into the vector pC1neo (Promega) and the plasmid transfected into HEK293 cells (ATCC). Stably expressing cell clones were identified after selection with G418 (1 mg ml^{-1}) using the [¹²⁵I]CGP64213 binding assay. The cells were grown to confluency on 15-cm tissue culture dishes. For cAMP-assays, cells were first washed, then detached with Krebs-Tris buffer containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX) and subsequently incubated at 37 °C for 20 min. About 10^5 suspended cells were transferred to prewarmed (37 °C) tubes and $20 \mu\text{M}$ forskolin plus test agents were added for 20 min. The cells were collected by centrifugation and lysed by the addition of 1 ml 70% ethanol, 7 mM HCl. cAMP concentrations were measured using a kit (Amersham).

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Structure of the adenylyl cyclase catalytic core

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Mammalian adenylyl cyclases contain two conserved regions, C₁ and C₂, which are responsible for forskolin- and G-protein-stimulated catalysis. The structure of the C₂ catalytic region of type II rat adenylyl cyclase has an α/β class fold in a wreath-like dimer, which has a central cleft. Two forskolin molecules bind in hydrophobic pockets at the ends of cleft. The central part of the cleft is lined by charged residues implicated in ATP binding. Forskolin appears to activate adenylyl cyclase by promoting the assembly of the active dimer and by direct interaction within the catalytic cleft. Other adenylyl cyclase regulators act at the dimer interface or on a flexible C-terminal region.

3',5' Cyclic AMP (cAMP), the prototypical second messenger, is central to hormone responses and many other biological processes^{1,2}. Adenylyl cyclases (EC 4.6.1.1), the sole source of cAMP, play a pivotal role in signal transduction following stimulation of G-protein-coupled hormone receptors. All of nine cloned mammalian isoforms of adenylyl cyclase are activated by the G protein α -subunit (G_{so}), and all but type IX are activated by the diterpene forskolin. In contrast, adenylyl cyclases are quite variable in their responses to G_i, G_o, and G_z α -subunits, G-protein $\beta\gamma$ subunits, Ca²⁺/calmodulin, protein kinase C and other regulators²⁻⁶. The primary structures of mammalian adenylyl cyclases consist of twelve hydrophobic membrane-spanning regions, the short loops that link them, and two cytoplasmic regions which contain both variable and conserved sequences³⁻⁸. The catalytic activity of the mammalian adenylyl cyclases is conferred by two well conserved domains that are also homologous to each other, designated C₁ and C₂ (refs 4-6, 9). Related catalytic domains occur in more than 30 different proteins; they are responsible for the catalytic activity of the guanylyl cyclases and many of the bacterial and fungal adenylyl cyclases⁴⁻⁶.

The complex transmembrane nature of adenylyl cyclases has so far precluded the solution of the three-dimensional structure of the intact enzyme. Regulated catalytic activity can be reconstituted at high levels in a soluble form by tethering the C₁ and C₂ regions^{9,10} with a linker, or by expressing the domains individually and then mixing them^{11,12}. The C₂ region by itself also has forskolin-stimulated catalytic activity, albeit greatly reduced compared to native adenylyl cyclase^{9,13}. Certain C₂-region variants have excellent solubility properties, which enables biophysical analysis to be simplified compared with intact adenylyl cyclases, tethered C₁ and C₂ hybrids, or C₁/C₂ mixtures. Because the underlying tertiary structure of all C₁ and C₂ domains is expected to be very similar, we have focused on the isolated C₂ region as a tractable model for adenylyl cyclase catalytic-domain structure. We now report the crystal structure of a rat type II adenylyl cyclase C₂-region variant (IIC₂- Δ 4; residues 871-1,090 (refs 13, 14) in complex with its potent activator, forskolin.

Adenylyl cyclase fold and quaternary structure

The structure of the adenylyl cyclase C₂ region was determined by multiple isomorphous replacement (Table 1 and Fig. 1) and has been refined to an *R*-factor of 21.9% against anisotropic data to 2.2 Å in the best direction and to 2.8 Å in the worst. The crystallographic refinement is equivalent to an isotropic diffraction limit of 2.58 Å based on equivalent integrated volumes of reciprocal space.

The overall structure of the adenylyl cyclase C₂ region monomer consists of a three-layer α/β sandwich (Fig. 2a). The structure contains a single large mixed β -sheet with strand order 2314(5/6)87 (Fig. 2b). Strands 5 and 6 run in opposite directions and are hydrogen-bonded to the second and first halves of the long strand 4, respectively. The β -sheet is antiparallel except for strands 4 and 6. Most of the β -sheet (strands 2, 3, 6, 7, 8, and half of strands 1 and 4) comprises the central layer of the sandwich. Strands 1 and 4 are doubled back on themselves by roughly 100 degrees to form part of the first and innermost layer. Strand 5 and helix 4 make up the rest of the first layer. The third and outermost layer consists primarily of two long helices, α 2 and α 3. Four regions, 871-874, 957-964, 1,059-1,071 and 1,077-1,090 could not be located reliably in the electron density for either molecule and were presumed to be partially or completely disordered. Manual inspection of the SCOP data base¹⁵ and extensive automated searching with DejaVu¹⁶ did not locate any related folds among available protein structures. We therefore believe that the adenylyl cyclase family contains a new protein fold.

The two C₂ region monomers are arranged in a wreath (Fig. 2c) in which the ends of each sandwich are bent towards each other and intertwine in head-to-tail fashion. The monomers are related to each other by a non-crystallographic local two-fold axis. The dimer interface buries 1,500 Å² of solvent-accessible surface area (1.4 Å probe¹⁷) per monomer. The centre of the wreath contains a dramatic cleft that is 15 Å wide by 15 Å deep by 25 Å long on the front or 'ventral' side. There is a shallower 8 × 12 × 28 Å groove on the 'dorsal' side. The cleft and the groove nearly converge in a tunnel at the local two-fold, which is blocked only by the isoleucine residue at position 940 (Ile940) from each monomer. The interactions between the two C₂ monomers are so extensive that there can be little doubt that this dimer corresponds to that observed in solution^{11,12}.

Two C₂ dimers are related to each other by the (110) crystallographic two-fold axis, forming a tetramer of approximate 222 symmetry (Fig. 2d). The dimers contact each other through their dorsal sides, whereas the ventral faces are nearly devoid of intermolecular contacts. The tetramer contacts are relatively polar and bury an average of only 470 Å² of solvent-accessible surface area/monomer. The oligomeric state of native membrane adenylyl cyclase is unknown. A native dimer, if it existed, would contain a total of two C₁ and two C₂ domains. If native adenylyl cyclase were a dimer or higher-order oligomer, it is uncertain whether the C₂ domain dimer we observe would correspond to an intra- or intermolecular association. The relevance of the crystallographic tetramer for understanding the native oligomer remains to be tested.

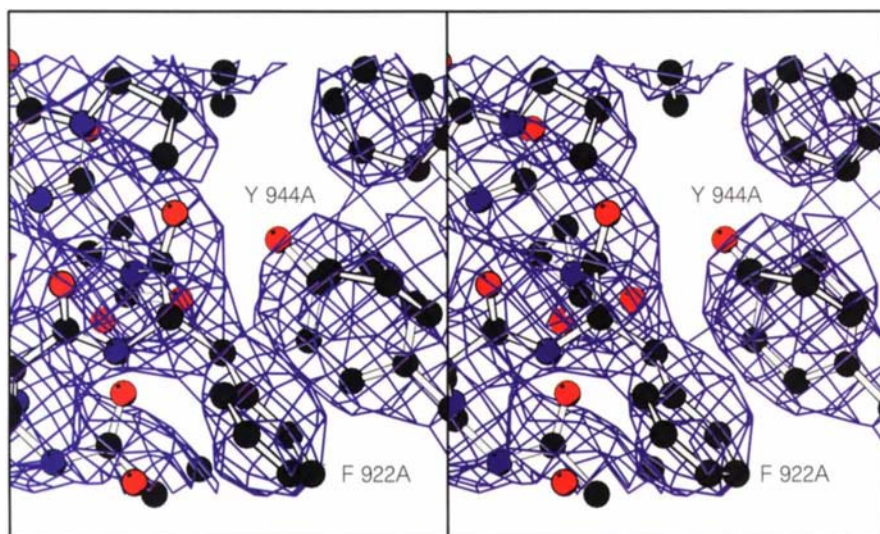


Figure 1 Experimental multiple isomorphous replacement density at 2.7 Å resolution contoured at 1.4 σ following density modification and phase extension. The refined structure is superimposed^{46,47}.

Catalytic site

The centre of the ventral cavity is lined with highly conserved polar and charged residues whose side chains point towards each other across the two-fold axis (Fig. 2e). These include Arg 997, Asn 1,025, Ser 1,028, Arg 1,029, Asp 1,031 and Ser 1,032 (Fig. 3). Mutations of the counterparts of some of these residues in type I adenylyl cyclase have a spectrum of disruptive effects on the binding of ATP and ATP analogues^{18,19}. In particular, Asn 1,025 and Arg 1,029 are essential for catalysis¹⁹. Adenylyl cyclases are subject to potent inhibition by certain ATP analogues known as P-site inhibitors²⁰. Some mutations alter the strength of ATP and ATP-analogue binding to the same extent, but often the effects differ. ATP analogue inhibitors might therefore bind to different conformations of the same site, have overlapping but non-identical binding sites, or have additional binding sites. The type I adenylyl cyclase C₁ domain (I-C₁) counterpart of R997A increases the half-maximal inhibitory concentration (IC₅₀) for the ATP analogue 2'-deoxy 3'-AMP by 24-fold. With the striking exceptions of Asn 1,025 and Arg 1,029, most mutational effects on activity are modest. Most of the mutants with significant effects on catalysis and ATP and ATP-analogue binding map to the walls of the ventral cleft. We conclude that the ventral cleft probably contains the binding sites for both ATP and its analogues.

The last ordered residues in the model are at the far corners of the ventral surface (Fig. 2a, b). The carboxy-terminal region is implicated in ATP binding by mutagenesis of the type I counterpart of Lys 1,067¹⁸, although the decrease in affinity is only eightfold. The type I peptide corresponding to residues 1,057–1,090 is the only site of reaction with the photo-activatable probe 8-azido-ATP²¹. The biochemical evidence for a role of the C-terminal sequence in ATP binding is seemingly at odds with its partial disorder in the crystal structure. The dilemma would be resolved if the C terminus were to become ordered over the ventral cavity upon ATP binding. There is a precedent for such a mechanism in the disorder–order transition in the flap of the Rous sarcoma virus protease²² on inhibitor binding.

The C₁ and C₂ regions of adenylyl cyclase have fundamentally different functional properties, despite their overall structural similarity. C₂, but not C₁, domains are active in the absence of the other, and isolated C₂ domains are far less active than native adenylyl cyclases or C₁–C₂ combinations. Some of these differences are likely to be due to replacement of key cleft residues in C₁ relative to C₂ (Fig. 3). These replacements include N1025T, S1028N, R1029X (where X is some other amino acid), D1031E and S1032X.

Forskolin binding site

The hypotensive diterpene forskolin from the Indian plant *Coleus*

forskolii has been useful pharmacologically for investigating adenylyl cyclase function because it activates nearly all mammalian cyclase isoforms^{23,24}. Although it probably does not participate in physiological cAMP signalling, its exceptional potency makes it important to understand its mode of action. The crystal structure of the IIC₂–forskolin complex shows that forskolin binds in two almost equivalent pockets at either end of the ventral cavity (Fig. 4a, b). This is consistent with the finding in solution that two sites on native type II adenylyl cyclase react with forskolin 6-isothiocyanate²⁵. Each pocket is formed by portions of α 1 and α 2 of one monomer; portions of β 1, β 3, β 5 and α 4 of the other monomer; and by the meeting of the β 2– β 3 turns from both monomers.

Prominent hydrogen bonds are made between O1 and O11 of the A forskolin and the peptide backbone of β 5A; O1 and the carboxylate of Asp 1018A; and the 7-acetyl group and both the main-chain and side-chain of Ser 942B (Fig. 4a, b). O9 is in a tightly packed region without any hydrogen bonds to protein, but is within hydrogen-bond distance of O1. The O6 points into the centre of the main cavity and is one of the few solvent-accessible forskolin atoms. The two hydrogen bonds between the protein and the O1 hydroxyl proton explain why 1,9-dideoxy forskolin and other derivatives at the 1-position²⁶ have little to no activity. The relatively open packing around O6 explains why forskolins derivatized at this position are active²⁶ and functional as photoaffinity labels of adenylyl cyclases²⁷. The dual interactions of the 7-acetyl group with Ser 942 are consistent with the observation that 7-acylation is important, although not essential, for forskolin activity²⁶.

The forskolin-binding pockets are overwhelmingly hydrophobic. Each contains a total of ten aliphatic and aromatic side chains within 4.5 Å of the forskolin (Fig. 4a, b). Each forskolin buries 90%, or 490 Å², of its solvent-accessible surface area upon binding. The binding site is highly hydrophobic, with 78% of the surface burial involving forskolin carbon atoms. Cyclase monomers A and B contribute equally (± 10 Å²) to binding each forskolin molecule. Forskolin stimulation of the soluble C₁ and C₂ regions of adenylyl cyclase correlates with their dimerization under certain conditions¹². The structure reveals that forskolin creates a direct hydrophobic bridge between the monomers. Each protein monomer contains two concave hydrophobic surfaces at the forskolin binding sites (Fig. 4c). The concavity of these surfaces prevents them from making direct hydrophobic contact with each other, and they form a crevice instead. By filling the gap and eliminating the highly unfavourable hydration of a concave hydrophobic surface²⁸, the forskolin bridge is expected to stabilize the dimer strongly.

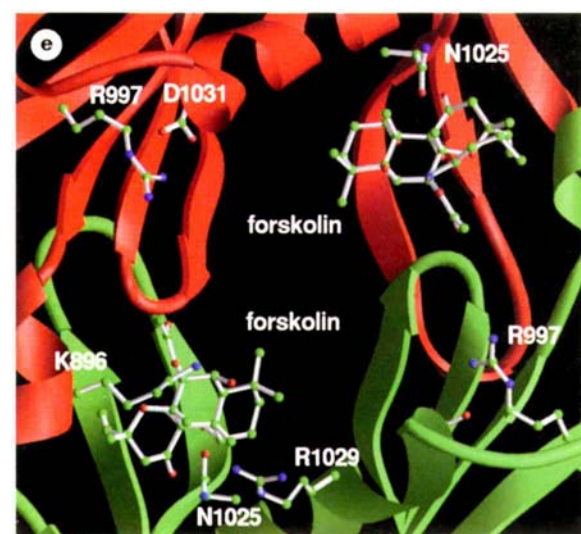
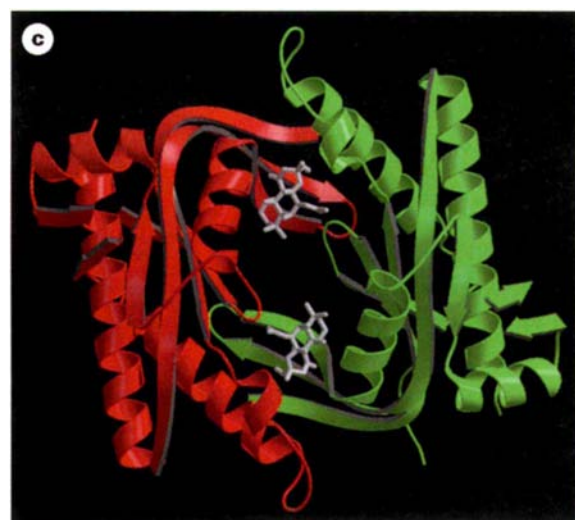
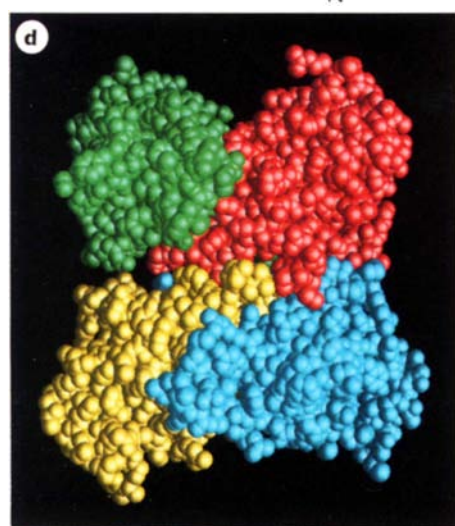
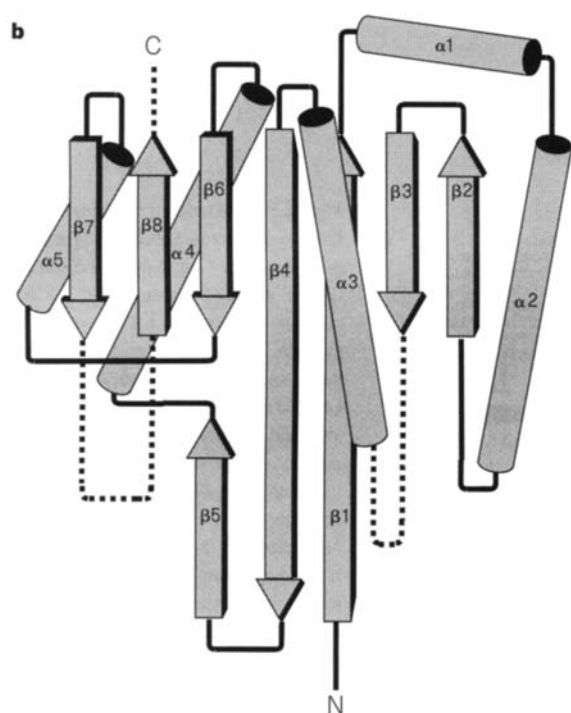
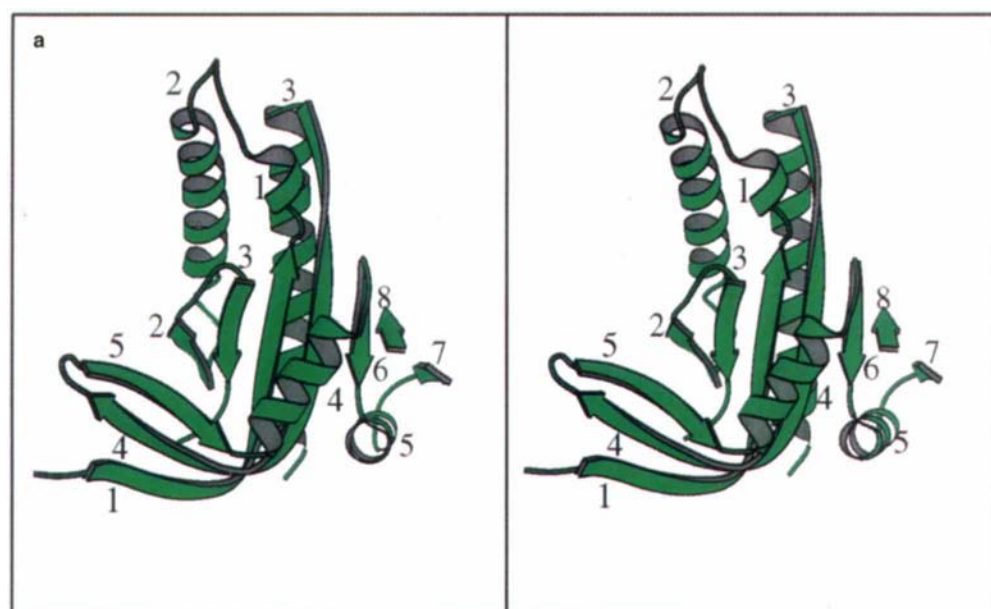


Figure 2 **a**, Stereoview of the overall structure of the adenylyl cyclase monomer⁴⁷. **b**, Schematic of the adenylyl cyclase catalytic-domain fold. In three dimensions, strand 5 and the bottom halves of strands 1 and 4 are bent around the back of the rest of the β -sheet. **c**, Catalytic dimer, with forskolin shown⁴⁸. **d**, Tetramer, with first dimer in red and green, second dimer in blue and magenta. **e**, Close-up view of putative ATP-binding residues in the ventral cleft.

Table 1 Experimental data on crystal structure determination and refinement

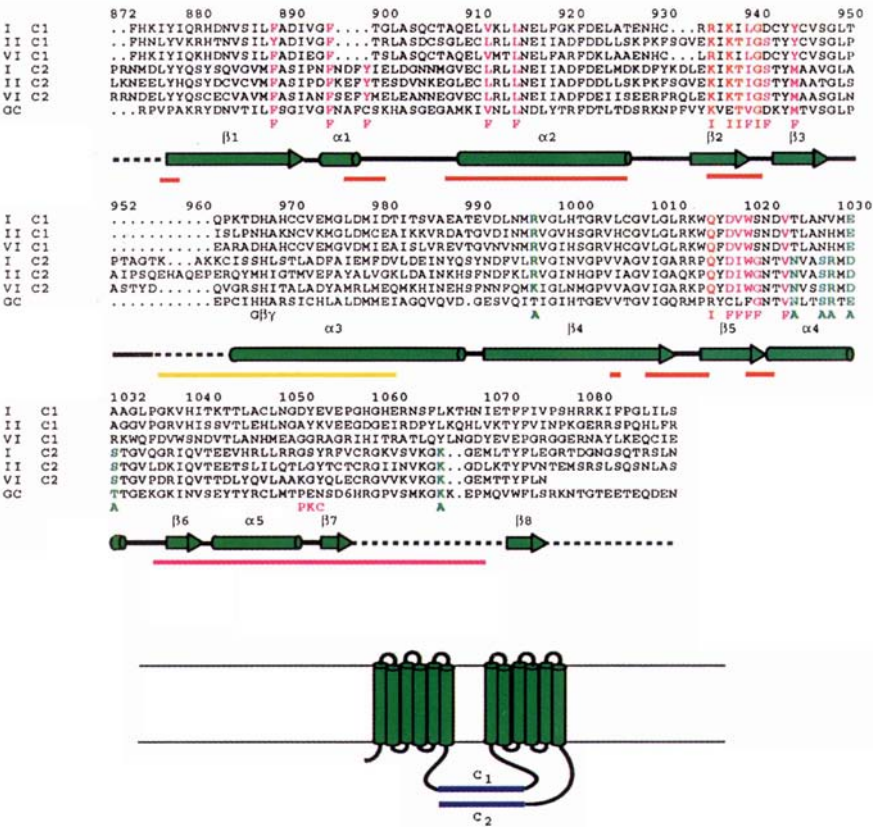
Data set	Resolution (Å)	R_{merge} (%)	No. of unique reflections	Total observations	Completeness (%)	R_{cullis} (%)	Phasing power	No. of sites
Native I	2.7	16.0	16,739	76,085	95.3	-	-	-
Native II	2.2	9.5	28,997	107,430	90.9	-	-	-
Native II aniso	2.2	6.7	17,326	51,618	54.2	-	-	-
UO ₂ Ac ₂ I	3.0	12.8	11,946	34,742	92.5	61.6	2.00	4
UO ₂ Ac ₂ II	3.0	15.0	12,307	85,261	97.5	65.2	1.69	4
Se substitution	3.5	16.6	7,369	23,455	88.2	67.6	0.82	8
(CH ₃) ₃ PbAc	3.5	14.9	7,330	28,359	88.7	73.6	0.63	4
UO ₂ Ac ₂ I ano	3.0	10.3	19,162	34,513	82.0	-	1.82	4
UO ₂ Ac ₂ II ano	3.0	12.3	20,683	55,431	90.6	-	1.87	4
(CH ₃) ₃ PbAc ano	3.5	13.2	11,966	28,319	81.4	-	0.84	4

Refinement (native II aniso)

Resolution (Å)	6.00	4.19	3.54	3.17	2.92	2.73	2.58	2.46	2.36	2.27	All 2.2
No. reflections	2,679	2,604	2,507	2,303	1,939	989	531	257	89	9	14,696
R -factor	0.185	0.184	0.230	0.272	0.285	0.285	0.305	0.328	0.311	0.373	0.219
Free R -factor	0.264	0.253	0.286	0.327	0.325	0.332	0.386	0.310	0.412	0.568	0.284

$R_{\text{merge}} = \sum ||_i - \langle I \rangle| / \sum I_i$, with Bijvoet pairs treated as equivalent. Total observations, the number of full and partial observations measured with non-negative intensity to the indicates resolution (except for 'aniso') or within the anisotropic ellipsoid of revolution ('aniso'). Completeness, the percentage of possible unique reflections measured with $I/\sigma(I) \geq 0$ to the indicated resolution. $R_{\text{cullis}} = \sum ||F_{\text{PH}} \pm F_{\text{P}}| - F_{\text{H}}| / \sum |F_{\text{PH}} \pm F_{\text{P}}|$ summed over centric reflections. Phasing power = $\langle F_{\text{H}} \rangle E_{\text{rms}}$ or $\langle F_{\text{A}} \rangle E_{\text{rms}}$ for isomorphous and anomalous differences. No. reflections, the number of reflections used in refinement for each resolution bin. R -factor = $\sum |F_{\text{o}} - F_{\text{c}}| / \sum F_{\text{o}}$ for all amplitudes with $F/\sigma(F) > 2$ (from native II aniso data set) measured in the indicated resolution bin; the free R -factor is calculated with 5% of the data in each bin.

Figure 3 Secondary structure and sequence alignment of C₁ and C₂ regions at bovine type I, rat type II and mouse type VI adenyllyl cyclases, and the 70K subunit of bovine soluble guanylyl cyclase. GenBank accession codes for these sequences are M15579, M80550, M96653 and Y00770, respectively. Dimer interface regions are underlined in red, protein kinase C response region in magenta, Gβγ-binding site in yellow. Forskolin binding residues of II-C₂ are highlighted in magenta, putative ATP-binding residues in green, and asymmetric interfacial residues red. Disordered regions are indicated by a dashed line. Residue numbers above the alignment correspond to the II-C₂ sequence and are aligned with the first digit.



Hydrophobic ligand binding complements engineered packing defects in destabilizing cavity mutants of T4 lysozyme²⁹ and the GCN4 leucine zipper³⁰. This striking effect in a natural regulatory system suggests that the forskolin-binding pocket might bind an as-yet unidentified physiological hydrophobic activator²⁴.

The forskolin-binding sites share the ventral cleft with the predicted catalytic site. The forskolin molecules are 8.2 Å apart at their closest point of approach (C18), leaving enough room in between for at least one nucleotide molecule. Forskolin makes direct contact with the catalytically essential residue Asn 1,025 and might help to position it. The possibility that forskolin directly contacts ATP cannot be ruled out. The likelihood that forskolin shares the cleft with ATP suggests a second activating role for forskolin which differs from its promotion of C₁/C₂ dimerization.

Other regulatory sites

Type II adenylyl cyclase is activated by the G-protein βγ subunit, which binds to the sequence 956–982 (refs 31, 32) (Fig. 5). The first part of this sequence corresponds to the first disordered section between β3 and α3. The second portion of the βγ binding site falls on the α3 helix, on the outermost layer of the overall structure.

Type II adenylyl cyclase is activated by protein kinase C (ref. 7). The region from 1,034–1,068 is essential for protein kinase C activation, although its role has been mysterious in that it does not contain a protein kinase C phosphorylation site³³. This region of the structure extends from strand 6 through the disordered C-terminal region. The first half of the protein kinase C response region is ventrolateral. It is near but ventral to the βγ binding site. The second half overlaps with the putative ATP-binding region within the flexible C-terminal region. Likewise, Ca²⁺/calmodulin regulates type I adenylyl cyclase by binding to a sequence immedi-

ately after the C₁ counterpart of the II-C₂ carboxy terminus^{34,35}. These observations suggest that the active conformation of the C-terminal region could be affected via the response region and the calmodulin-binding site.

Subunit interfaces and asymmetry

In spite of the overall symmetric appearance of the dimer, there are marked differences between the two domains in the interfacial regions. The main-chain atoms of 25 interfacial residues, which were not subjected to non-crystallographic restraints during refinement, deviate in position by 2.0 Å r.m.s. A few side chains, such as Lys 938, point in opposite directions when superimposed. This asymmetry addresses, in part, the flaw in the C₂ homodimer as a model for natural adenylyl cyclases—a chemically symmetric species replaces an asymmetric one. The asymmetry in the dimer suggests that the adenylyl cyclase catalytic domains may have a propensity to form asymmetric structures. In addition to differences in the cleft residues of the C₁ and C₂ regions, the asymmetric dimer interface may also be a factor in the differences between C₂ homodimers as opposed to C₁/C₂ heterodimers and native enzyme.

Mutagenesis shows that catalysis is sensitive to the structure of the interface. The point mutant that most drastically perturbs ATP-analogue binding, with a 260-fold increase in IC₅₀ (ref. 18), is the I-C₂ counterpart of Lys 938. This mutation also perturbs ATP binding. Lys 938 is in the bottom of the dorsal groove on top of the local two-fold axis, and its side-chain position is completely different in the two monomers. The location of Lys 938 precludes its interaction with ligands in the main ventral cleft. It might be asked how this mutant leads to such extraordinary effects when it appears to be outside the catalytic cleft. It is unlikely that ATP binds in the dorsal groove near Lys 938, because of steric constraints and because this

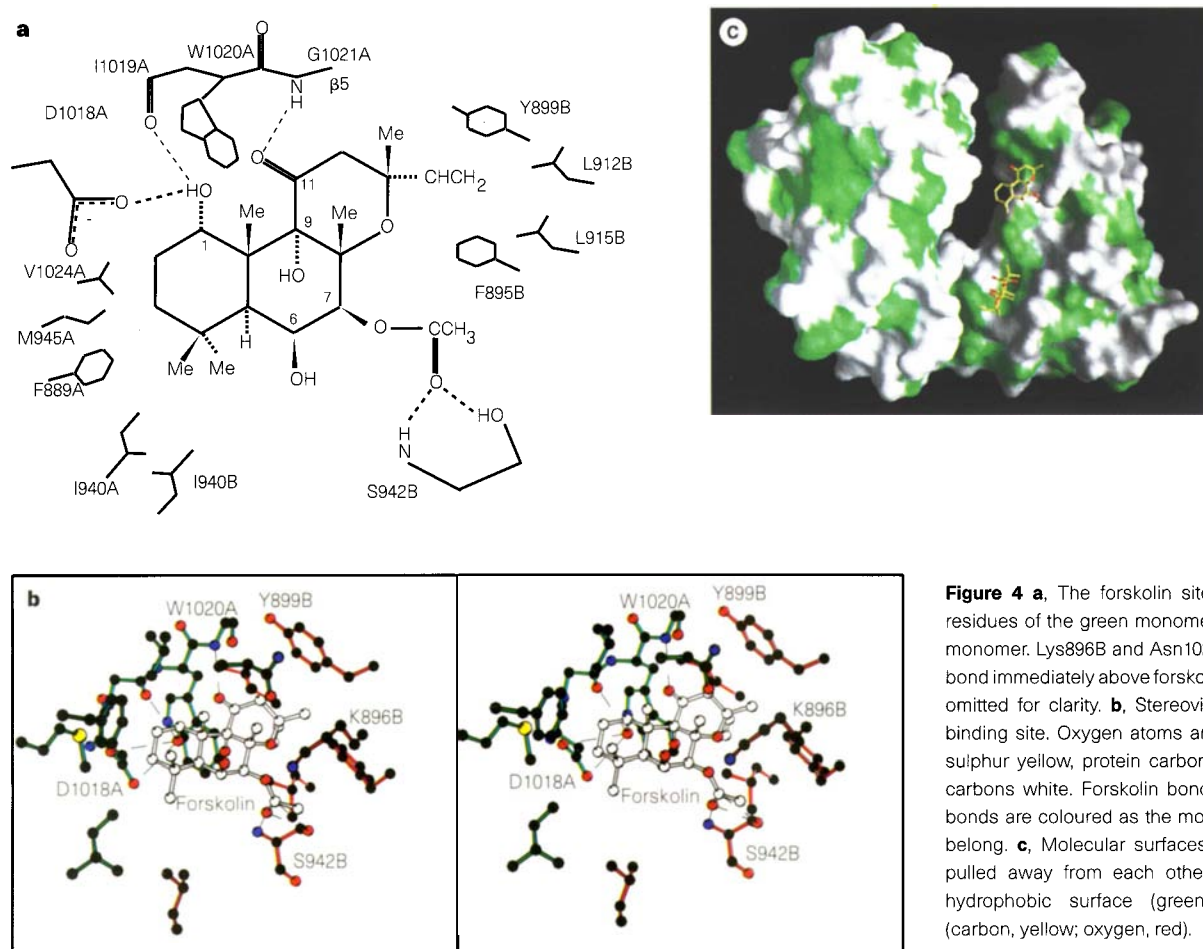


Figure 4 a. The forskolin site. Suffix A refers to residues of the green monomer, suffix B to the red monomer. Lys896B and Asn1025A form a hydrogen bond immediately above forskolin in this view but are omitted for clarity. **b.** Stereoview of one forskolin-binding site. Oxygen atoms are red, nitrogen blue, sulphur yellow, protein carbon black and forskolin carbons white. Forskolin bonds are white, protein bonds are coloured as the monomer to which they belong. **c.** Molecular surfaces⁴⁹ of C2 monomers, pulled away from each other to reveal concave hydrophobic surface (green) binding forskolin (carbon, yellow; oxygen, red).

would not explain the finding that many ventral-cleft mutants perturb ATP binding. It is more likely that the dimer interface at the two-fold axis is a focal point for regulating the stability and alignment of the dimer. Furthermore, random mutagenesis of *Dictyostelium* adenylyl cyclase has produced a collection of catalytically inactive and G-protein-insensitive mutations³⁶. Of the mutations that fall within the C₁/C₂ regions, about half map to β 4 and the β 4– β 5 turn in or adjoining the interface.

Several potential metal-binding sites occur at the subunit interfaces. Ca²⁺ is an important regulator of many of the adenylyl cyclases, although its effects on type II adenylyl cyclase are mediated by calmodulin and protein kinase C (refs 5–7). Adenylyl cyclase is markedly activated by Mn²⁺ in preference to Mg²⁺, although it is not known whether or not this action of Mn²⁺ is at the active site. One class of metal-binding sites were located by uranyl derivatization, which occurred in three regions. The first is where Asp 894, Glu 897, Glu 901, Asp 903 and Glu 907 from one monomer meet a symmetry-related copy of the second monomer. This subunit pairing is unlikely to be physiological because it is not found in either the dimer or tetramer. The second is formed at the functional dimer interface, where Glu 875 of one monomer approaches Glu 917, Asp 921 and Asp 925 of the other. The third is an intra-subunit site near but outside the cleft, and consists of Asp 984, Asn 987, Asp 993 and Asp 1,037.

Two clusters of tightly bound solvent molecules are found in the dimer interface on either side of the local two-fold axis, where the β 2– β 3 turn, the β 4– β 5 turn and β 5 strands of each monomer meet each other. Principal ligands in cluster I are the main-chain carbonyl oxygens of 939B, 941B and 1,017A, and the side-chain oxygens of Thr 939A, Gln 1,016A, and Asp 1,018A. The side-chain of 938A also approaches cluster I. The key cluster-II ligands are the main-chain carbonyl of 937A and the side chains of Lys 938B and Gln 1,016B. The differences in the two clusters highlight the marked asymmetry in the interfacial interactions and illustrate the key role of this part of the structure in forming both the interface and the floor of the active site. The observation that cluster-I water ligands are all oxygens suggests that this site could also bind divalent metal ions. The details of steric packing around clusters I and II appear too tight to allow access by an ATP molecule. If they indeed bind metal ions,

the function of these sites is likely to be regulatory rather than catalytic.

Activation mechanism

The dimeric adenylyl cyclase catalytic core structure confirms one proposed activation mechanism, while revealing others that are entirely new. The most dramatic finding is that the active site is formed jointly by both catalytic domain monomers upon dimerization. Dimerization is stabilized, in turn, by the forskolin hydrophobic bridge. This bears out the concept that adenylyl cyclase activation is coupled to a shift in equilibrium favouring the association or intramolecular dimerization of C₁ and C₂ domains in the native enzyme^{11,12}.

The structure of the catalytic core complexed with forskolin reveals that the forskolin-binding sites share the principal cleft with the putative catalytic site. Forskolin is more intimately involved with the enzyme active site than was suspected. It makes direct contact with the catalytically important Asn 1,025, suggesting a possible mechanism for enhancing catalysis. Direct or water-mediated interactions between forskolin and the substrate itself cannot be ruled out.

We have found that a portion of the C-terminal region of adenylyl cyclase, which has been proposed to interact with ATP in solution, is disordered in the crystal structure in the absence of ATP. This region overlaps a region required for protein kinase C activation in type II adenylyl cyclase, and its C₁ counterpart adjoins the calmodulin-binding domain of type I adenylyl cyclase. These observations support the idea that conformational plasticity of the carboxy terminus of the C₁ or C₂ regions could be exploited by multiple regulatory mechanisms in adenylyl cyclase isoforms. Finally, metal binding sites at the interfaces could mediate both intra- and intermolecular associations of adenylyl cyclase catalytic domains.

In summary, the structure of the catalytic core of the adenylyl cyclases allows many different regulatory mechanisms to operate simultaneously. It is perfectly suited to their physiological roles as coincidence detectors of multiple signals. □

Methods

Protein crystallization. Native and selenomethionyl rat type II adenylyl cyclase residues 871–1,090 (IIC₂- Δ 4)¹³ were expressed as N-terminal polyhistidine fusions using pProEX-HA6-IIC₂- Δ 4 and purified from bacterial lysates by immobilized metal-ion-chelating chromatography¹², cleaved with thrombin (1 U per mg protein) overnight at 4 °C (ref. 13), and purified on a FPLC Mono-Q column using a NaCl gradient. IIC₂- Δ 4 (17.5 mg ml⁻¹) was crystallized at 21–24 °C in the presence of 1 mM forskolin, 2% DMSO, 50 mM Tris-HCl, 80 mM NaCl, 10 mM DTT, 1.1–1.2 M (NH₄)₂SO₄, 0.1 M sodium phosphate, pH 5.80–5.90, in space group *P*₄₃₂₁₂, *a* = 81.3, *c* = 180.5 Å. Heavy-atom derivatives were prepared by soaking crystals for 4 h (I) or 40 h (II) in 2.0 mM uranyl acetate and for 10 h in 1.0 mM trimethyllead acetate, both in modified mother liquor lacking phosphate buffer. Crystals were flash-frozen after brief incubation in modified mother liquor with 20% glycerol.

Structure determination and refinement. Data were collected in the laboratory on an R-Axis IIC detector (native I and derivatives) and at the Cornell High Energy Synchrotron Source at beamline A1 (λ = 0.908 Å) using image plates (native II), and integrated and merged with the HKL package³⁷. Uranium positions were located by Patterson search with HASSP³⁸ and other heavy-atom sites were located in cross-phased difference Fouriers using the PHASES package³⁹. Heavy-atom parameter refinement was carried out with PHASES using maximum likelihood. Solvent flattening (solvent fraction, 0.55) and two-fold non-crystallographic symmetry averaging were carried out with dm^{40,41}, and phases were extended to 2.7 Å. The sequence of rat type II adenylyl cyclase¹⁴ was fitted to the electron density with program O (ref. 42), which was facilitated by the use of (SeMet-Native) difference syntheses to identify methionine positions. The structure was refined with XPLOR 3.8 (ref. 43) using non-crystallographic symmetry restraints on 77% of atoms. The current model includes residues 877A–956A, 875B–951B and 965–1,058 and 1,072–1,076 of both monomers, 2 forskolin molecules, and 120 water molecules. The

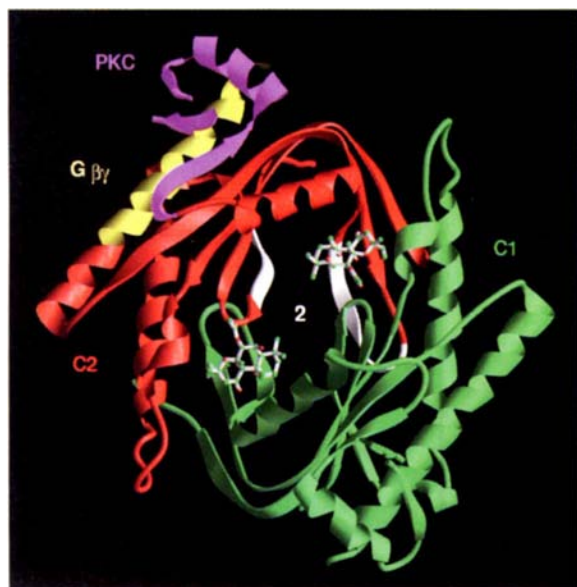


Figure 5 G β γ -binding site (yellow), protein kinase C response region (magenta), and asymmetric zone including Lys938 (white) at the local two-fold axis ('2')⁵⁰. The green monomer represents the presumed location of the C₁ domain on the basis of its homology to the C₂ domain.

assignment of amino acids 1,072–1,076 to $\beta 8$ is tentative owing to the high level of disorder in the C-terminal region. Because of the markedly anisotropic diffraction, data used in refinement were truncated according to an ellipsoid of rotation with axes along a^* and b^* of $1/(2.8 \text{ \AA})$, and along c^* of $1/(2.2 \text{ \AA})$ (native II aniso). This yielded 14,696 unique reflections used in refinement with $(F/\sigma(F) > 2)$ between $6.0 \text{ \AA}$ and the upper limit. This ellipsoid covers a volume of reciprocal space equal to that of a sphere with an isotropic diffraction limit of $2.58 \text{ \AA}$. R.m.s. deviations of bonds and angles from ideal values⁴⁴ are $0.011 \text{ \AA}$ and 1.5 degrees, respectively. The r.m.s. coordinate deviation for all 2,114 atoms restrained to obey non-crystallographic symmetry is $0.075 \text{ \AA}$. The working and free (5%) R -factors for data $(F/\sigma(F) > 2)$ from $6.0 \text{ \AA}$ to the anisotropic high-resolution limit are 0.219 and 0.284 , respectively. Stereochemical values are all within or better than the expected ranges for a $2.6 \text{ \AA}$ structure⁴⁵. Asp903B, which is in the highly mobile $\alpha 1$ – $\alpha 2$ linker, is the only non-glycine residue in a disallowed region of the Ramachandran plot.

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Correspondence and requests for materials should be addressed to J.H.H. Coordinates have been deposited in the protein data bank at Brookhaven with accession code 1AB8; prerelease coordinates will be made available at <http://www-mlmb.niddk.nih.gov/hurleygroup.html>.

Chaotic variations in the eccentricity of the planet orbiting 16 Cygni B

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The planet recently discovered¹ orbiting the star 16 Cyg B has the largest eccentricity ($e = 0.67$) of any known planet. Planets that form in circumstellar disks are expected to have nearly circular orbits, although gravitational interactions in a system of two or more planets could generate high-eccentricity orbits^{2,3}. Here we suggest that the eccentric orbit of 16 Cyg Bb arises from gravitational interactions with the distant companion star, 16 Cyg A. Assuming that 16 Cyg Bb formed in a nearly circular orbit, with the orbital plane inclined between 45° and 135° to the orbital plane of 16 Cyg A, and that there are no other planets with a mass similar to that of Jupiter within 30 astronomical units (AU, the average distance between the Earth and the Sun), then 16 Cyg Bb will oscillate between low-eccentricity and high-eccentricity orbits. The transitions between these orbits should occur every 10^7 – 10^9 years, with the planet spending up to 35 per cent of its lifetime with an eccentricity $e > 0.6$. These results imply that planetary orbits in binary stellar systems commonly experience periods of high eccentricity and dynamical chaos, and that such planets may occasionally collide with the primary star.

A numerical integration of the equations of motion of the planet illustrates the oscillation of eccentricity and inclination (Fig. 1). The short-period curves are for binary periastris distance $q_b = 100$ AU; the long-period curves are for $q_b = 500$ AU. Over an oscillation period the eccentricity smoothly increases from zero to $e = 0.75$, and returns. In turn, the orbital inclination decreases to $i = 40^\circ$, and returns. The range of the oscillations changes little over several oscillation periods, and is almost the same in both integrations. We suggest that 16 Cyg Bb is currently in the high-eccentricity phase of this oscillation.

For further exploration we simplify the problem. The evolution of the orbital shape and orientation is slow compared to the orbital period of either the planet or the binary star. Thus, following Gauss⁴, we can 'smear' the companion into an elliptical ring and compute the secular evolution of the planet orbit under the gravitational force from this ring⁵. Gauss's method is four orders of magnitude faster than direct integration, but accurately reproduces the orbital evolution shown in Fig. 1. This increase in computation speed allows us to explore the evolution of the planet over longer times and a wider range of parameter space.

Using Gauss's method we simulated the behaviour of 1,000 'planets' with initial semimajor axis $a_0 = 1.7$ AU and initial eccentricity $e_0 = 0.001$, distributed on a grid of angular elements (inclination, argument of perihelion, ascending node). Each planet was integrated for 10^4 binary periods or until its eccentricity exceeded 0.99. Figure 2 shows the maximum eccentricity attained by each planet and the fraction of time that each surviving planet spent with $e > 0.6$, as functions of initial inclination i_0 . For $i_0 < 39^\circ$, the maximum eccentricity remains low. At $i_0 = 39^\circ$ there is a sharp transition, and the maximum eccentricity approaches unity as the inclination increases. Figure 2 also shows that highly inclined planets can spend up to 35% of their lives with $e > 0.6$.

This behaviour can be explained analytically⁶. The time-averaged potential of the distant secondary star at the planet is

$$\Phi = \frac{GM_s}{4[a_b q_b (1 + e_b)]^{3/2}} (2z^2 - x^2 - y^2) [1 + O(ae_b/q_b)] \quad (1)$$

where $x, y, z \approx a$ are the cartesian coordinates and semimajor axis of the planet, measured from the primary star with z normal to the binary orbit, M_s is the mass of the secondary star, and $a_b, e_b, q_b = a_b(1 - e_b)$ are the orbital elements of the binary. If we keep only the lowest order in a/q_b , then Φ is axisymmetric, and the binary eccentricity affects only the numerical coefficient of the potential and not its spatial dependence (thus the form of the potential is the same if the binary orbit is circular or nearly parabolic). We next average Φ over the planetary orbit. In this approximation, $\Theta = (1 - e^2) \cos^2 i$ is an integral of motion (Kozai's integral). Only one degree of freedom survives in this simplified model, so trajectories in phase space follow contours of Φ . These are best described in terms of the dimensionless momentum variable $x = 1 - e^2$ and the angle conjugate to it, ω , the argument of perihelion. Figure 3 shows these contours for $\Theta = 0.25$ ($i = 60^\circ$ when $e = 0$). The phase space is divided into a region in which the argument of perihelion ω circulates and one in which ω librates about $\pi/2$. Consider a planet that starts near $x = 1$ ($e = 0$) and $\omega = 0$. As time elapses, the planet's eccentricity is forced to large values, reaching a maximum when $\omega = \pi/2$, and then returning to the initial value. As Θ is conserved, i must decrease when e increases, and vice versa. This is what we observe in Fig. 1. As Θ is increased (initial inclination decreased) the libration point shifts upward, and the libration island shrinks—disappearing for $\Theta \geq 3/5$ —and trajectories are not forced to large eccentricities. At $e = 0$,

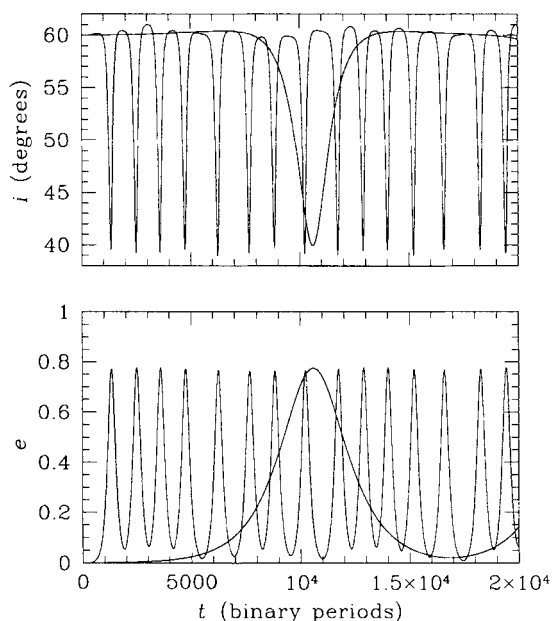


Figure 1 The inclination i (top panel) and eccentricity e (bottom panel) of the planet versus time, as determined by a numerical integration of the three-body equations of motion. We assume that initially 16 Cyg Bb orbits 16 Cyg B, with elements $a = 1.7$ AU, $e = 0.001$ and $i = 60^\circ$ with respect to the orbital plane of the binary (the median inclination if the orbital planes are uncorrelated). The masses M_p and M_s of the primary and secondary star were assumed to be $1M_\odot$, consistent with their near-solar spectral types, and the mass of 16 Cyg Bb was assumed to be 1.5 Jupiter masses. The binary semimajor axis was assumed to be $a_b = 1,000$ AU. The short-period curves are for binary periastris distance $q_b = 100$ AU; the long-period curves are for $q_b = 500$ AU. The range of planetary eccentricity and inclination is independent of a_b and q_b ; only the timescale of the oscillation is affected by a_b and q_b . The planet spends roughly 25% of its time with $e > 0.6$.

$\Theta = 3/5$ corresponds to $i = \cos^{-1}(3/5)^{1/2} = 39.2^\circ$, coinciding with the transition point in Fig. 2.

When the assumptions of the averaged model are relaxed, the trajectories are free to wander from the contours. The scattered dots in Fig. 3 are the data from the numerical integration with $q_b = 100 \text{ AU}$ in Fig. 1. Although no averaging has been done and the terms in Φ that we have dropped are $O(ae_b/q_b) \approx 2\%$, the dots still follow the contours. Near the separatrix, the trajectory is now permitted to swap back and forth from circulation to libration. This alternation is diagnostic of chaos. The trajectory we show is in fact chaotic, with a Lyapunov time of $\sim 9 \times 10^7 \text{ yr}$. All initially circular orbits are forced into the vicinity of the separatrix; thus it is likely that the orbit of 16 Cyg Bb—and planets formed in similar binary systems—is chaotic.

The averaged model can be used to estimate (1) the maximum eccentricity attained by a trajectory and (2) the fraction of time spent at high eccentricity. Given the initial eccentricity and inclination at $\omega = 0$, (1) may be obtained by computing e when $\omega = \pi/2$, and (2) may be found by integrating the time spent with $e > 0.6$ over an oscillation period. The solid and dashed lines in Fig. 2 show these results, for initial eccentricities $e_0 = 0.001, 0.01$ and 0.1 . At initial inclination $i_0 > 39.2^\circ$, the maximum eccentricity is insensitive to e_0 and is given by $e = (1 - (5/3) \cos^2 i_0)^{1/2}$. On the other hand, the time spent with $e > 0.6$ is quite sensitive to e_0 ; in practice, the terms of $O(ae_b/q_b)$ in equation (1) lead to a small forced eccentricity, which renders the long-term evolution insensitive to small values of e_0 . (We note that models discussing some aspects of the eccentricity oscillation of planets in binary systems have been developed independently by Mazeh *et al.*¹¹ and Innanen *et al.*¹²).

We can now estimate the probability of observing a planet with an

eccentricity above a given value. Assuming that the initial orbital plane of the planet is randomly oriented with respect to the binary plane and that semimajor axes of the planet is much smaller than that of the binary, the probability of observing such a planet is

$$P(e > e^*) = \int_0^{\pi/2} f(e^*, e_0, i_0) \sin i_0 di_0 \quad (2)$$

where $f(e^*, e_0, i_0)$ is the fraction of time spent with $e > e^*$. We find that between 10% and 25% of planets in suitable binary systems would show $e > 0.6$, assuming e_0 is between 0.001 and 0.2.

The validity of our explanation for the high eccentricity of 16 Cyg Bb depends on three related conditions. First, the orbital planes of the planet and the binary must be initially uncorrelated. This is a natural assumption because the equatorial planes of solar-type stars in binaries are not aligned with the binary orbital plane when the binary semimajor axis exceeds 40 AU (ref. 7), far smaller than the semimajor axis in 16 Cyg; the inner and outer orbital planes in hierarchical triple stars are correlated⁸, but observational selection limits the period of the outer orbit to $\lesssim 200 \text{ yr}$ ($a \lesssim 34 \text{ AU}$), far smaller than in 16 Cyg. Second, the eccentricity oscillation half-period must be less than the age of the system. The oscillation period depends on Kozai's integral Θ and on the location in the phase plane, but is given roughly by

$$P_{\text{precess}} \approx P_{\text{orb}} \left(\frac{M_p}{M_s} \right) \left(\frac{a_b}{a} \right)^3 (1 - e_b^2)^{3/2} \quad (3)$$

$$\approx 10^8 \text{ yr} \left(\frac{P_{\text{orb}}}{1 \text{ yr}} \right) \left(\frac{a_b}{1,000 \text{ AU}} \right)^{3/2} \left(\frac{q_b}{100 \text{ AU}} \right)^{3/2} \left(\frac{1 \text{ AU}}{a} \right)^3$$

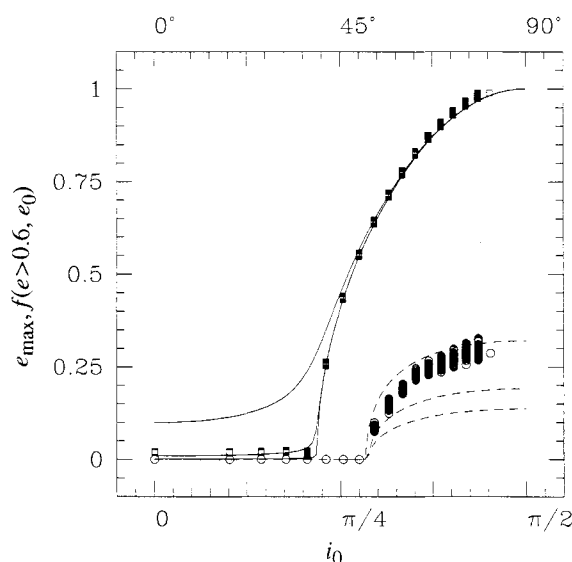


Figure 2 The open squares mark the maximum eccentricity, e_{max} , attained by an ensemble of simulated planets, as a function of initial inclination i_0 . The planets had initial semimajor axis and eccentricity of $a_0 = 1.7 \text{ AU}$ and $e_0 = 0.001$. The binary semimajor axis and eccentricity were $a_b = 1,000 \text{ AU}$ and $e_b = 0.9$. The open circles mark the fraction f of time the planet spent with $e > 0.6$. Only the surviving planets are shown. For each quantity we show the predictions of our analytic model, for three initial eccentricities $e_0 = 0.001, 0.01, 0.1$. At high initial inclinations, the maximum eccentricity (solid lines) is insensitive to e_0 , but the time fraction (dashed lines) depends strongly upon e_0 .

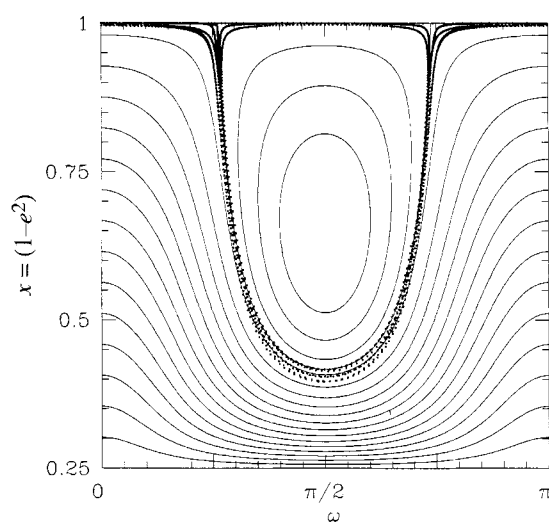


Figure 3 Phase space trajectories for $\Theta = 0.25$. The horizontal axis shows the argument of perihelion, ω , and the vertical axis shows the conjugate variable, $x = 1 - e^2$. The phase space trajectories from $\omega = \pi$ to 2π are identical. The scattered points are the results of a single three-body integration with $q = 100 \text{ AU}$ (shown in Fig. 1). It is clear that the averaged model captures the overall dynamics except that motion near the separatrix is chaotic.

where P_{orb} is the orbital period of the planet and M_p is the mass of the primary star. We have assumed $q_b/a_b = 1 - e_b \ll 1$ and $M_p \approx M_s$ in the second equality. This is less than the age for most plausible binary orbits.

A third condition is that the companion must provide the dominant contribution to the apsidal precession of the planet; otherwise its effect on the eccentricity averages to zero as the apsis precesses. Because the precession time⁵ is long, this is a stringent requirement, which can be violated by contributions to the precession, such as: (1) a hypothetical second planet or remnant disk associated with the primary star. The apsidal precession period due to a planet or disk of mass M_x at semimajor axis $a_x \gg a$ is roughly $1 \times 10^6 \text{ yr} (P_{\text{orb}}/1 \text{ yr}) (a_x/10 \text{ AU})^3 (1 \text{ AU}/a)^3 (M_{\text{Jupiter}}/M_x)$. If this is to exceed the period in equation (3) then we strongly constrain the properties of other planets or a disk in 16 Cyg B; for example, there cannot be another Jupiter-mass planet with semimajor axis $\leq 30 \text{ AU} (a_b/1,000 \text{ AU})^{1/2} (q_b/100 \text{ AU})^{1/2}$. (2) General relativistic effects. The apsidal precession period due to relativistic corrections to the newtonian equations of motion is⁹

$$P_{\text{gr}} = \frac{2\pi c^2 (1 - e^2) a^{5/2}}{3(GM_p)^{3/2}} = 3.36 \times 10^7 \text{ yr} (1 - e^2) \left(\frac{P_{\text{orb}}}{1 \text{ yr}} \right) \left(\frac{a}{1 \text{ AU}} \right) \left(\frac{M_{\odot}}{M_p} \right) \quad (4)$$

where c is the speed of light and $M_{\odot}$ is the solar mass. If this is to exceed the period in equation (3) for 16 Cyg Bb, we must have $(a_b/1,000 \text{ AU})(q_b/100 \text{ AU}) \leq 3$.

Extra-solar planets also reside in the binary systems 55 Cancri ($a = 0.11 \text{ AU}$, $e = 0.051$) and τ Boötis ($a = 0.0462 \text{ AU}$, $e \approx 0$). Why are these orbits nearly circular? The circularization time due to tides raised in the planet by the primary star is probably less than the age in τ Boo but longer than the age in 55 Cnc (ref. 10); thus the near-circular orbit of at least 55 Cnc must be explained. Although 55 Cnc may simply be in the low-eccentricity phase of its oscillation, there is a better explanation. The binary separation in 55 Cnc is $1,150 \text{ AU}$; assuming $a_b = 1,150 \text{ AU}$, $q_b = 337 \text{ AU}$ ($e_b = 1/\sqrt{2}$, the theoretically expected median value), and $M_p = M_s = M_{\odot}$, we find $P_{\text{precess}} \approx 2 \times 10^{10} \text{ yr}$ and $P_{\text{gr}} = 6.7 \times 10^4 \text{ yr}$. Thus relativistic precession is much faster than companion-induced precession, and high eccentricities cannot develop, even if there are no other planets in the system.

Another consequence of eccentricity oscillations is that up to $\sim 1\%$ of planets in binary systems (those with initial inclinations near 90°) will eventually collide with the primary star if no other process intervenes. For planets on initially circular orbits in randomly oriented planes, the fraction whose minimum perihelion is $< q$ in our analytic model is $f = (6q/5a)^{1/2} = 0.075(q/R_{\odot})^{1/2} (1 \text{ AU}/a)^{1/2}$ where $R_{\odot}$ is the solar radius. However, this result neglects relativistic precession, which increases rapidly with eccentricity, thereby setting an upper limit to the eccentricity than can be reached via secular evolution in many systems. $\square$

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Evidence for magnetic polarons in the magnetoresistive perovskites

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Manganese perovskites based on the compound LaMnO_3 are attracting considerable theoretical and technological interest by virtue of their unusual magnetic and electronic properties^{1–4}. Most notable of these properties is the extremely large change in resistivity that accompanies the application of a magnetic field, an effect known as ‘colossal’ magnetoresistance. The origin of this effect has been attributed^{5–7} to the presence of magnetic polarons—charge carriers accompanied by a localized (and magnetically polarized) distortion of the surrounding crystal lattice^{8,9}—but their existence and properties remains a matter of speculation. Here, using a combination of volume thermal expansion (with and without an applied field), magnetic susceptibility and small-angle neutron scattering measurements, we present evidence for the existence of magnetic polarons above the ferromagnetic ordering temperature, T_c . We detect the spontaneous formation of localized ~ 12 -Å magnetic clusters above T_c which, on application of a magnetic field, grow in size but decrease in number. We argue that the response of these magnetic polarons to an applied magnetic field underlies the pronounced magnetoresistive properties in the compounds $(\text{La}_{1-x}\text{A}_x)_{2/3}\text{Ca}_{1/3}\text{MnO}_3$ (where A is Y or Tb).

Giant magnetoresistance effects in Mn perovskites have recently been observed at temperatures close to the insulator–metal transition temperature⁷. This electronic transition is accompanied by a paramagnetic–ferromagnetic phase transition at the same temperature (T_c). Much effort has been devoted to understand the ground state in the ‘paramagnetic state’ and the mechanism which drives the insulator–metal transition. Activated semiconductor-like conductivity above T_c suggested the possibility of conduction by polarons in the paramagnetic regime⁶. Neutron scattering^{10,11} and magnetization measurements¹² pointed to short-range magnetic correlations well above T_c . We will argue here that magnetostructural and magnetoresistive measurements, along with small-angle neutron scattering (SANS) experiments under magnetic field, in two different series of compounds showing giant magnetoresistance, $(\text{La}-\text{Y/Tb})_{2/3}\text{Ca}_{1/3}\text{MnO}_3$, give a picture where the carriers become localized as the lattice is distorted and magnetically polarize the neighbouring manganese atoms above T_c . The lattice ‘small polaron’ was originally proposed by Holstein¹³. The polaron described in our work coincides with the ‘small polaron’ description, with the addition of magnetic polarization resulting from short-range ferromagnetic interactions. (The related concept of magnetic polarons unaccompanied by a lattice distortion was introduced by Kasuya and Yanase¹⁴ to explain the anomalous transport phenomena in Eu-chalcogenide alloys and further applied to magnetic semiconductors^{15–17}.)

The lattice distortions produced by the polaronic effect in our polycrystalline samples is detected by measuring the volume thermal expansion. For that, the techniques used were⁸: above room temperature, a push rod and differential transformer method; and below room temperature, the strain-gauge technique. Both methods

allow measurements of how the volume of the crystal lattice changes with temperature. In the absence of additional effects, the obtained curves should correspond to the Grüneisen anharmonic phonon contribution. The change in volume when a magnetic field is applied (the volume magnetostriction, ω) has also been measured with the strain-gauge technique. The presence of short-range magnetic correlations (due to the ferromagnetic double-exchange interaction¹⁸) in the 'paramagnetic state' can be detected by measurements of magnetic susceptibility, χ . These measurements were performed in a SQUID magnetometer. The deviation of the inverse susceptibility, $1/\chi$, from the high-temperature straight line corresponding to non-interacting magnetic moments (Curie–Weiss

behaviour) marks the onset of the magnetic interaction between magnetic moments. Electrical resistivity was measured by the four-point technique. For the magnetoresistivity measurements the magnetic field was provided by a commercial superconducting coil.

The SANS experiments were performed at the high-flux reactor of the Institute Laue-Langevin (ILL), in Grenoble, France. The instrument used, D16, uses a neutron wavelength of 4.5 Å and allows neutron momentum transfers, q , ranging between 0.03 and 0.6 Å⁻¹. For the SANS measurements under magnetic field a superconducting magnet was used. In crystalline materials, the typical SANS intensity is comprised of two signals. One has a magnetic origin (changing with temperature and magnetic field)

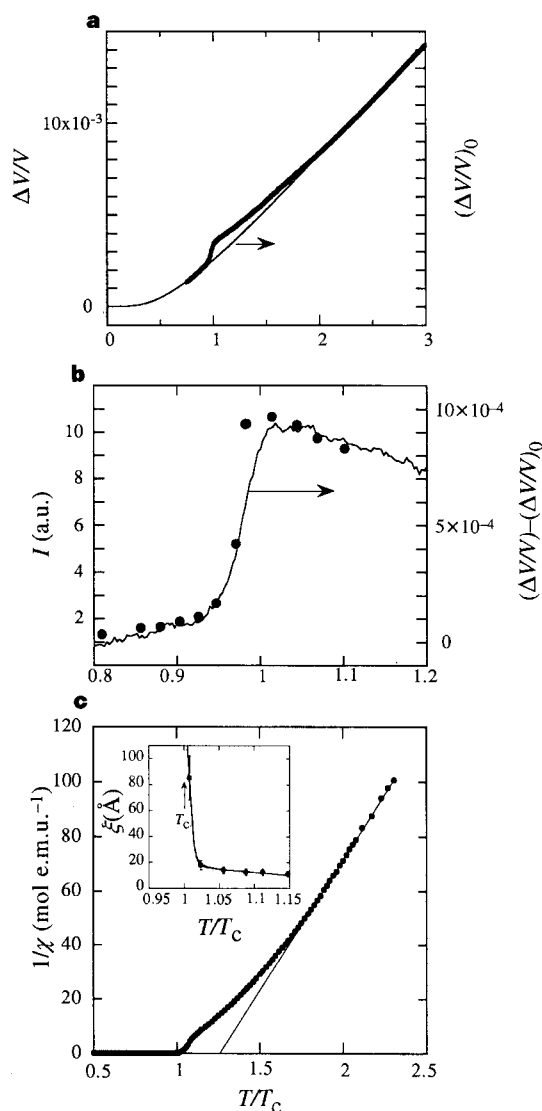


Figure 1 **a**, Experimental volume thermal expansion, $(\Delta V/V)$, and the calculated anharmonic Grüneisen-like phonon contribution, $(\Delta V/V)_0$, for $\text{La}_{2/3}\text{Ca}_{1/3}\text{MnO}_3$. Both curves separate at $\sim 1.8T_c$ and coincide again at T_c . **b**, Anomalous volume lattice distortion $(\Delta V/V) - (\Delta V/V)_0$ (solid line), ascribable to the lattice distortions caused by the polarons, and the magnetic SANS intensity (large dots) around T_c . **c**, Thermal dependence of the inverse magnetic susceptibility, $1/\chi$. The straight line is a fit to the Curie–Weiss law, $1/\chi = C/(T - \Theta)$ resulting in $\Theta = 1.25T_c$ and C corresponding to the expected value for free manganese magnetic moments. Deviation from the Curie–Weiss law at around $1.8T_c$ signals the onset of short-range ferromagnetic correlations. Inset, the magnetic correlation length, ξ , as a function of temperature.

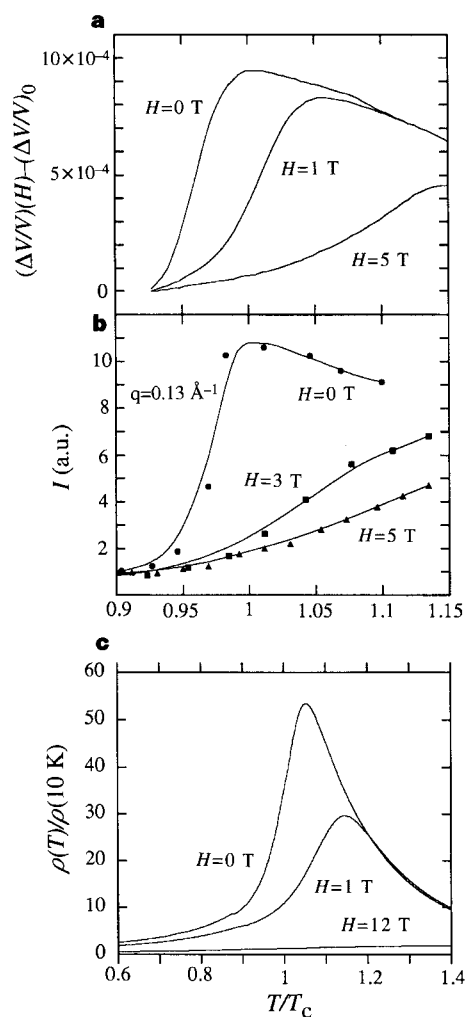


Figure 2 Behaviour under applied magnetic field of: **a**, the difference $(\Delta V/V)(H) - (\Delta V/V)_0$, which indicates that the lattice distortion decreases with the applied magnetic field; **b**, the magnetic SANS intensity, which correlates with the volume distortion; **c**, the normalized resistivity for $\text{La}_{2/3}\text{Ca}_{1/3}\text{MnO}_3$, showing activated behaviour above T_c and the insulator–metal transition at around T_c .

and the other is due to incoherent nuclear scattering (constant with temperature and field). In polycrystalline materials SANS scattering from a small grain size is also possible. In our experiments this last possibility is ruled out because of the grain size and q range involved (The grain size in these samples¹² is typically 10^3 – 10^4 Å, which would give a SANS contribution at $q \approx 10^{-3}$ – 10^{-4} Å⁻¹. This q range is far below the range used in these experiments.) To obtain the magnetic SANS intensity the incoherent nuclear scattering (which is very small in the studied q range) was determined and subtracted. The magnetic correlation length, ξ , of the ferromagnetic clusters (namely, its size) can be obtained by fitting the SANS intensity to a Lorentzian-type q dependence ($I = I_0/(q^2 + \kappa^2)$,

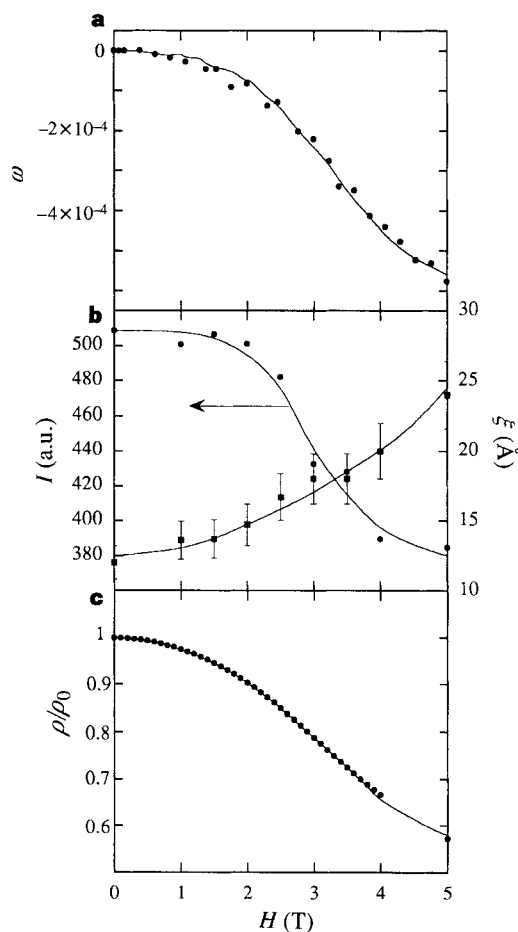


Figure 3 Behaviour at $T = 1.1T_c$ as a function of the magnetic field of: **a**, volume magnetostriction, $\omega = (\Delta V/V)(H) - (\Delta V/V)(H = 0)$, obtained by measuring the striction parallel ($\lambda_{||}$) and perpendicular ($\lambda_{\perp}$) to the applied magnetic field and calculating $\omega = \lambda_{||} + 2\lambda_{\perp}$; **b**, magnetic SANS intensity I (at $q = 0.0357$ Å⁻¹) and correlation length, ξ , of the ferromagnetic clusters; **c**, resistivity normalized to the value at zero field for $\text{La}_{2/3}\text{Ca}_{1/3}\text{MnO}_3$. Lines are visual guides. The resemblance between the curves in the three panels is evidence for magnetoresistivity due to magnetopolaron effects.

where $\kappa = 1/\xi$). The SANS intensity is proportional to the number of scattering centres¹⁹ (in other words, to the number of small clusters) provided that they do not overlap.

In Fig. 1a we have plotted the experimental results of the volume thermal expansion, $\Delta V/V$, and the calculated Grüneisen anharmonic phonon contribution, $(\Delta V/V)_0$, for a Debye temperature of $\Theta_D = 500$ K (refs 5, 20). One can see that at temperatures below $\sim 1.8T_c$ (~ 500 K) an extra anharmonic contribution appears. This anomalous contribution to the volume lattice distortion increases gradually as the temperature is lowered to T_c , where a sharp volume contraction of $\sim 0.1\%$ takes place and the Grüneisen contribution is recovered. The lattice distortion observed in this temperature range is associated with the gradual localization of the charge carriers below $\sim 1.8T_c$ (refs 8, 21) due to a strong electron–phonon coupling, resulting in a strong influence on the lattice vibrations^{3,9}. The mechanism for conduction is thermal-activated polaron hopping. At T_c the lattice distortion disappears because at the insulator–metal transition the carriers become delocalized. In Fig. 1b, together with the extra volume distortion (experimental minus phonon contribution curves) as a function of temperature, we show the magnetic contribution to the SANS at a momentum transfer of $q = 0.13$ Å⁻¹. The large magnetic SANS intensity above T_c indicates the presence of small ferromagnetic clusters which are abruptly suppressed at T_c . It is remarkable that both curves superimpose, suggesting the same underlying physical mechanism for both effects. Evidence for the existence of this magnetic clustering effect was also obtained from the large short-range ferromagnetic contribution observed in the measurement of the magnetic susceptibility above T_c (Fig. 1c). At around $1.8T_c$, $1/\chi$ deviates from Curie–Weiss behaviour. In the inset of Fig. 1c the magnetic correlation length obtained from the SANS fits is shown. The rapid divergence of ξ at T_c due to magnetic critical fluctuations is another indication of the magnetic origin of the observed SANS intensity. Above T_c the determined size of the magnetic clusters is ~ 12 Å, varying slightly with temperature. In all of the analysed cases of the series $(\text{La–Y/Tb})_{2/3}\text{Ca}_{1/3}\text{MnO}_3$, at zero magnetic field and below $T \approx 2T_c$, magnetic clusters of linear size between 8 and 20 Å appear. These results will be published in detail elsewhere.

The volume thermal expansion under different magnetic fields is shown in Fig. 2a. The anomalous volume thermal expansion, which is associated with the charge localization, is largely reduced by the magnetic field. The role of the magnetic field is to enhance the hopping of the carriers between different lattice sites. Carrier localization is consequently disfavoured and the anomalous volume lattice distortion disappears. One can see in Fig. 2b that the SANS intensity decreases with magnetic field in the same manner as the volume thermal expansion does. This seems to indicate that the number of ferromagnetic clusters decreases but the spatial extension of the remaining clusters grows because the magnetic field tends to align the manganese magnetic moments. This is reflected in an increase in the magnetic correlation length (as will be shown later). In Fig. 2c we show the resistivity ρ as a function of temperature under different applied magnetic fields. At zero field, the increase in ρ as T_c is approached from above is due to gradual carrier localization. At T_c , the carrier is delocalized and a rather sharp (the sharpness depends on the sample quality, that is, grain size and T_c inhomogeneity) insulator–metal transition takes place. The magnetic field strongly decreases the resistivity, bringing about giant magnetoresistance ratios.

In Fig. 3 we show measurements (at $1.1T_c$) of volume magnetostriction, SANS intensity and resistivity as a function of the magnetic field. Below $H \approx 1.5$ T, the different reported physical properties remain almost unaltered. But above $H \approx 1.5$ T, a rapid decrease in volume occurs. The same change can be seen in the SANS intensity, which rapidly decreases at the same magnetic field. The correlation length increases to reach a value of ~ 25 Å at $H \approx 5$ T. The resistivity drops to 57% of the initial value at $H \approx 5$ T.

Responsive gels formed by the spontaneous self-assembly of peptides into polymeric β -sheet tapes

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Molecular self-assembly is becoming an increasingly popular route to new supramolecular structures and molecular materials^{1–7}. The inspiration for such structures is commonly derived from self-assembling systems in biology. Here we show that a biological motif, the peptide β -sheet, can be exploited in designed oligopeptides that self-assemble into polymeric tapes and with potentially useful mechanical properties. We describe the construction of oligopeptides, rationally designed or based on segments of native proteins, that aggregate in suitable solvents into long, semi-flexible β -sheet tapes. These become entangled even at low volume fractions to form gels whose viscoelastic properties can be controlled by chemical (pH) or physical (shear) influences. We suggest that it should be possible to engineer a wide range of properties in these gels by appropriate choice of the peptide primary structure.

Our initial studies focused on a 24-residue peptide K24, whose primary structure (NH₂-Lys-Leu-Glu-Ala-Leu-Tyr-Val-Leu-Gly-Phe-Phe-Gly-Phe-Phe-Thr-Leu-Gly-Ile-Met-Leu-Ser-Tyr-Ile-Arg-COOH) is related to the transmembrane domain of the IsK protein⁸. This peptide, and a longer version of it (K27; ref. 9), readily form β -sheet structures in lipid bilayers, suggesting they would be good candidates for formation of β -sheet tapes in amphiphilic solvents. Indeed, we find that solutions of K24 in solvents such as methanol or 2-chloroethanol produce transparent viscoelastic gels at concentrations above 0.002 volume fraction ($\sim 3 \text{ mg ml}^{-1}$), stable up to the boiling point of the solvent. Infrared (IR; Fig. 1a) and circular dichroism (CD; Fig. 1b) spectra show that in methanol the peptide molecules have a predominantly antiparallel β -sheet conformation. The wide-angle X-ray diffraction of the gel reveals the presence of a 4.7 Å structural periodicity, consistent with the side-by-side separation expected for β -strands in β -sheet structures¹⁰. Transmission electron microscopy (TEM; Fig. 1c) reveals the presence of distinct, tape-like structures, $\sim 8 \text{ nm}$ wide (the length of the K24 molecule in the β -strand conformation) and with apparent lengths typically in excess of 0.1 μm .

The rheological properties of the gels provide further insight into their structure. The response to small-strain oscillatory shear (Fig. 2a) is typical of highly entangled polymer gels^{11,12}. From the elastic modulus G_N^0 we calculate, assuming entropic rubber elasticity^{11,12}, a mesh size of 10–100 nm for gels with peptide volume fractions 0.03–0.003 (see Methods for details). The stress response is seen to be linear up to strains of 230% (Fig. 2b). This permits an estimate of a lower limit of 13 nm or greater for the persistence length of the tape, indicative of a moderately rigid polymer, consistent with the intrinsic rigidity of β -sheet structures. We have deduced from the rheological data (see Methods) an upper limit of 0.7 nm for the thickness of a tape, consistent with tapes a single molecule

We propose that the only way to interpret all these measurements is to assume that in the 'paramagnetic state' the observed lattice distortions and ferromagnetic clusters are caused by the same entity—the magnetic polaron—which is responsible for the giant magnetoresistance effects. From the measurements at zero field we have shown that the two ingredients of the proposed magnetic polaron, a lattice distortion and a ferromagnetic cluster, are simultaneously present at temperatures of $T_c - 2T_c$. One could think that they are uncorrelated and exist independently. However, the measurements of the change in volume and SANS intensity under magnetic field indicate that the ferromagnetic cluster is associated with the lattice distortion (small polaron). From these experiments, we conclude that both effects are linked. This is deduced from the way the curves of the volume lattice distortion (Fig. 2a) and the SANS intensity (Fig. 2b) superimpose under fixed magnetic field and, more importantly, the way the volume and the SANS intensity behave at fixed temperature versus the applied magnetic field (Fig. 3a and b). The data in Fig. 3a and b are easily understood considering that the magnetic polarons retain their structure unchanged up to $\sim 1.5 \text{ T}$ (at $1.1T_c$); the lattice distortion and the associated ferromagnetic cluster remain unchanged (no variation in either the volume or the SANS intensity). For larger magnetic fields, a tendency towards carrier delocalization and an increase in the size of the associated ferromagnetic cluster occurs.

The observed field effect on the character and degree of localization of the charge carriers provides evidence for the close relation between the existence of these entities (referred to as 'magnetic polarons') and the giant magnetoresistive effect. We would like to put emphasis on this aspect considering the result shown in Fig. 3c. The field-dependence of the resistivity is very similar to that found for ω and the SANS intensity. As a consequence, the mechanism for the giant magnetoresistance effect is related to the existence and nature of the magnetic polarons. A simple description of the observed behaviour should consider the polaronic lattice effect provided by a narrow polaronic band ('small polaron', that is, strong electron–phonon interaction) along with the magnetic character of the carrier (ferromagnetic cluster) which would make possible a broadening of the polaronic band under the presence of an applied magnetic field. Further theoretical developments on the giant magnetoresistance effect in these manganese perovskites should consider these ideas. □

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thick. Atomic force microscopy also reveals tapes with thickness of 0.5–1 nm.

To elucidate the molecular interactions governing the self-assembly of peptides into β -sheet tapes, we have explored the behaviour of K24 as a function of the polarity ϵ_r and hydrogen bonding ability α of the solvent¹³. Our observations are represented as a 'phase diagram' in Fig. 3a. In solvents such as 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) having $\alpha \geq 1.5$, clear, low-viscosity solutions

comprising mixtures of helices and random coils are obtained, as shown by IR spectra (Fig. 1a) and far-ultraviolet CD spectra (Fig. 1b). By contrast, transparent, thermostable, self-supporting gels are formed in moderately polar solvents, such as methanol, with $25 < \epsilon_r < 68$, $\alpha < 1.5$, and peptide concentrations above 0.002–0.004 volume fraction (3–6 mg ml⁻¹). In less polar solvents ($15 < \epsilon_r < 25$) and in solvents with $\epsilon_r > 68$, the β -sheet tapes are not sufficiently soluble to form gels. Below $\epsilon_r \approx 15$, the proportion of peptide in the helical form increases. Addition of the surfactant SDS to the peptide gel formed in methanol increases the stability of the α -helix with respect to the β -sheet structure to such an extent that a simple newtonian fluid is obtained, demonstrating that the β -sheet structure is in part stabilized by attractive forces between hydrophobic side-chains in polar solvents¹⁴.

To impart gelation to more polar solvents, β -sheet tapes with hydrophilic surfaces are a prerequisite. This is demonstrated by the

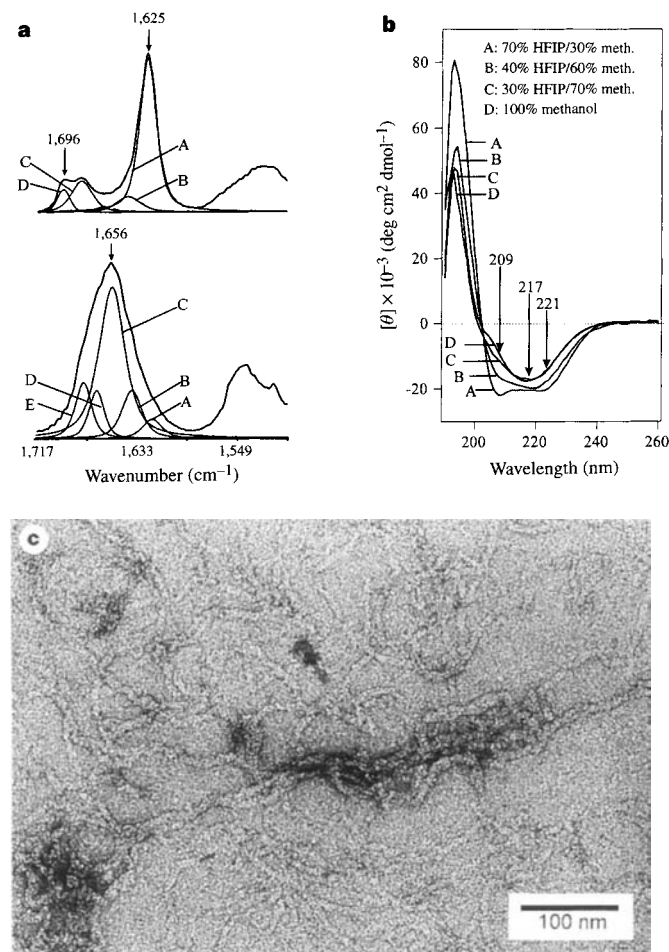


Figure 1 **a**, Band-fitted Fourier-transform IR spectra of amide I (1,700–1,600 cm⁻¹) and amide II (1,600–1,500 cm⁻¹) bands of K24 at volume fraction ~0.004 (~6 mg ml⁻¹). Component bands are labelled A–D. Top, gel in 100% methanol. Assignment of component bands: A 1,625 cm⁻¹, (maximum of amide I), β -sheet; B 1,641 cm⁻¹, random coil; C 1,680 cm⁻¹, residual trifluoroacetic acid (TFA); D 1,696 cm⁻¹, antiparallel β -sheet³³. The half-height bandwidth of the major component at 1,625 is 17 cm⁻¹. The peak in the amide II region at 1,530 cm⁻¹ is also consistent with a β -conformation. Bottom, spectrum of a clear, fluid solution of K24 in 90% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP)/10% methanol. Assignment of component bands: A 1,620 cm⁻¹ and B 1,638 cm⁻¹, β -sheet; C 1,655 cm⁻¹ (maximum of amide I), α -helix; D 1,667 cm⁻¹, turns; E 1,678 cm⁻¹, TFA. The half-height bandwidth of the major component at 1,655 cm⁻¹ is 26 cm⁻¹. The peak in the amide II band at ~1,545 cm⁻¹ is also consistent with the predominance of helical conformation. The amide II bands are wider than the amide I bands, as expected²². Amide II bands are slightly distorted due to imperfect subtraction of solvent spectrum. **b**, Far-UV circular dichroism spectra of K24 in HFIP/methanol mixtures at peptide volume fraction ~0.00006 (~0.081 mg ml⁻¹). $[\theta]$ is the mean residue molar ellipticity. **c**, Transmission electron micrograph, obtained as described in Methods section, showing a network of polymer tapes. The tapes are ~7–8 nm wide and are entangled. Very few free ends are apparent, consistent with very long tapes. The density of the tapes decreased as the solution was diluted. With prolonged exposure, the structures degraded.

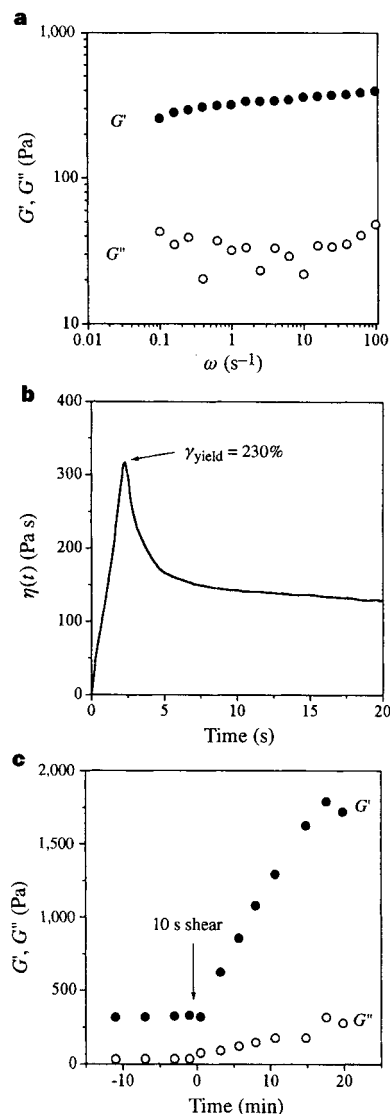


Figure 2 Viscoelastic properties of 0.019 volume fraction (~30 mg ml⁻¹) K24 in 2-chloroethanol at 25°C. **a**, Typical mechanical spectra (elastic modulus G' and viscous modulus G'' versus frequency ω of applied strain) of fully set gels with 1% strain (see Methods), which is within the linear viscoelastic region. **b**, Plot of the stress growth $\eta(t)$ versus time obtained with steady shearing of the sample at a shear rate of 1 Hz. **c**, Growth of the plateau elastic and viscous moduli after shearing for 10 s, at a constant shear rate of 5 Hz. Data were collected with small oscillatory deformation experiments (5% strain), at a shear rate ω of 1 s⁻¹.

behaviour of the peptide Lys β -21 whose structure (CH₃CO-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-NH₂) corresponds to residues 41–61 of hen egg white lysozyme and forms a triple-stranded β -sheet in the β -domain of the native protein¹⁵. The first strand of this domain is exposed to water. The 'phase diagram' (Fig. 3b) is very similar to the one for K24, except that gels are now obtained generally in solvents with $\epsilon_r > 30$, including, for example, water ($\epsilon_r = 78$) and formamide ($\epsilon_r = 109$). The IR spectrum of the gel is consistent with a predominantly β -sheet structure.

Criteria for the rational design of gel-forming peptides have been deduced from the above results and information in the relevant literature^{6,16–19}. In addition to the highly cooperative intermolecular hydrogen bonds, which are of primary importance for the generation of the tapes, these are: (1) cross-strand attractive forces (hydrophobic, electrostatic, hydrogen-bonding) between side-chains, (2) lateral recognition between adjacent β -strands to constrain their self-assembly to one dimension, and (3) strong adhesion of solvent to the surface of the tapes to control solubility. Based on these criteria, a *de novo* 11-residue peptide DN1, CH₃CO-Gln-Gln-Arg-Phe-Gln-Trp-Gln-Phe-Glu-Gln-NH₂, was designed to form β -sheet polymer tapes in water. Indeed, the peptide adopts a β -strand configuration in D₂O, as revealed by its IR spectrum (the major component of the amide I' band is centred at 1,619 cm⁻¹ and has half-height bandwidth of 20 cm⁻¹), and produces a self-sup-

porting, thermostable gel, up to at least 90 °C, and at volume fractions of 0.01 (~15 mg ml⁻¹) or above at neutral pH in water (see Methods for details). The hydrophobic interactions are provided by the (–CH₂–)₂ moieties of the six glutamine residues (Fig. 4). The residues Phe 4, Trp 6 and Phe 8 are also hydrophobic, but they are also expected to provide intermolecular recognition by π – π interactions^{18,20}. Arg 3 and Glu 9 provide an additional degree of recognition via their strong coulombic attraction¹⁸, and favour an antiparallel alignment of the strands. Gln, Arg and Glu side-chains make the β -sheet surface hydrophilic. Chemically blocked termini were used to avoid edge-to-edge coulombic attractions between tapes. In contrast, an 11-residue peptide CH₃CO-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gln-NH₂ assembles into β -sheet structures which are highly insoluble in water, consistent with the behaviour of polyglutamine-based proteins²¹. We believe that this behaviour stems from the absence of lateral recognition between β -strands and/or strong intersheet interactions as compared with those to the solvent.

An exploitable property of the peptide gels is their responsiveness to chemical and physical triggers. For example, the Lys β -21 gels are stable up to pH 12, but are transformed into newtonian fluids at higher values, presumably as a consequence of deprotonation of the side-chains Arg 5 and Arg 21. Indeed, it is well known that peptide conformations can be switched by changes in the pH of the solution^{6,15,22}. A gel-to-fluid transition can also be brought about by varying the hydrogen-bond donor strength of the solvent^{13,23}. This can be achieved, for example, in the case of the K24/methanol system, by adding HFIP (see Figs 1b and 3a).

The novel rheological response of the K24 gel in 2-chloroethanol to a strong shear flow is an interesting example of a physical trigger (Fig. 2c). The elastic and viscous moduli are 'switched' over a timescale of 20 minutes into a state with a much higher modulus (by a factor of 5), but a much smaller linear range of strain (~2%, that is, a more brittle gel). Relaxation to the equilibrium state takes of the order of 10 hours. This unusual behaviour may be connected with annealing of flow-induced structural defects in the tapes. Such a phenomenon is not observed in linear polymers and other self-assembled polymers such as the cylindrical worm-like micelles formed by surfactants²⁴.

The β -sheet peptide tapes show some similarity, in structural terms, to protein fibrils formed from a variety of different proteins, including the polyglutamine-containing proteins responsible for Huntington's disease²¹ and the more complex structure of amyloid fibres¹⁰. Other linear biological peptides, such as leucine-rich (LRR) fragments of the *Drosophila* Toll protein²⁵, a 28-oligonucleotide fragment of the β -amyloid protein²⁶ and peptides modelled on conserved domains of desmin²⁷, as well as synthetic peptides incorporating non-natural chemical groups^{28–30} have been reported to form gels.

The peptide gels described here are potentially biocompatible and biodegradable and in this respect can be compared with biopolymer gels³¹ such as gelatin, actin, amylose and agarose. The rheological measurements on K24 indicate comparable mechanical properties (elastic and viscous moduli) to these 'classic' biopolymer gels under linear deformation, but show much greater recoverable strain. The mechanically induced strengthening of the peptide gels could be used to programme gel properties simply by shearing. Thus, we believe that the polymeric β -sheet tapes will find a wide range of applications, and will also enable fundamental issues to be explored. Particular issues are the elucidation of the molecular mechanism and control of β -sheet folding pathways in both functional native proteins and those responsible for amyloidogenesis in neurological diseases, as well as the physics of tape-like polymers³². □

Methods

IR. Spectra were averages of four scans, recorded with a resolution of 4 cm⁻¹, at 20 °C in a 50- μ m CaF₂ cell, using a Perkin Elmer 1760X FTIR spectrometer.

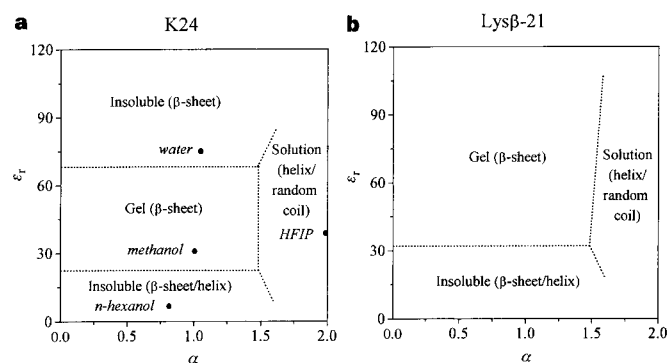


Figure 3 Conformational propensities and gelation properties of the peptides K24 (**a**) and Lys β -21 (**b**), as a function of the relative dielectric constant ϵ_r and the hydrogen-bond donor ability α of the solvent (peptide volume fraction ~0.006, corresponding to ~10 mg ml⁻¹). The α -values for different solvents were obtained by the solvatochromic comparison method¹³. Details of the solvents used are given in the Methods section. The areas separated by the dotted lines represent distinctive behavioural patterns. The four points shown for K24 in **a** are examples of representative solvents lying within each region. Preliminary results with the *de novo* peptide DN1 show a similar phase diagram to that of Lys β -21 (**b**), except that the gel band for the *de novo* peptide extends from $\epsilon_r \approx 40$ to $\epsilon_r \approx 90$, and the peptide becomes insoluble at $\epsilon_r > 90$.

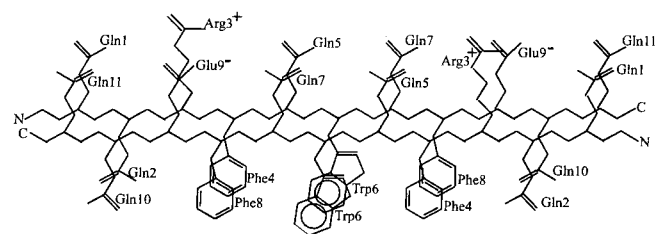


Figure 4 Proposed antiparallel β -sheet arrangement of two molecules of the *de novo* peptide DN1. The peptide backbones are drawn as black zig-zag lines. The N and C termini are indicated with the letters N and C, respectively. Side chains that carry a net charge at neutral pH are labelled with + or –.

Component peaks were obtained by second-derivative analysis and band-fitting of the absorption spectra³³, after subtraction of the solvent spectrum.

CD. Measurements were performed at 20 °C on a Jasco J-715 spectropolarimeter using quartz cuvettes (path length 1 mm) and a response time of 1 s. Each spectrum was the average of four scans. The peptide concentration in solution was determined by amino acid analysis and ninhydrin assay. CD curves were fitted using the algorithm CCA (ref. 15).

TEM. The specimens were prepared by deposition of the peptide gel (0.001 volume fraction or 1.5 mg ml⁻¹ in methanol, prepared 24 h before study to ensure complete formation of the gel and then diluted 20 times before use) onto a 300 mesh size, glow-discharged, carbon-coated, copper grid followed by negative staining with uranyl acetate solution (4% w/v in water). The samples were examined with a Phillips CM10 TEM at 100 kV accelerating voltage.

Rheology. A Rheometrics Dynamic Analyser II, with parallel plate (25 mm diameter) geometry, was employed. The thickness of the material between the plates was between 0.5 and 0.8 mm. Rubber elasticity theory^{11,12} has been used to extract information about the structure and dynamic properties of the gel network. For example, the average distance ξ between nearest entanglements in space (mesh size) is: $\xi = \{(g_N f_b T)/(2G_N^0)\}^{1/3}$ where G_N^0 is the elastic modulus in the plateau region, measured from the mechanical spectra in the linear viscoelastic region, $g_N \approx 1$, k_B is Boltzmann's constant, T the absolute temperature, and f , the number of network tapes associated with each entanglement, is taken to have a value ≥ 4 . The stress growth $\eta(t)$ of the material is monitored as a function of time, at constant shear rate $\dot{\gamma}$. The stress builds up proportionally to the duration of the applied shear. The strain at which the tapes are fully extended is $\gamma_{\text{yield}} \approx N_e^{1/2}$, where N_e is the number of persistence lengths of the tapes between nearest entanglements on the same tape. Further increase of the strain causes the network to break down and the stress to relax. Using the above equations for ξ and γ_{yield} , a lower limit can be placed on the persistence length $l \geq \xi/N_e^{1/2}$. The thickness, h , of a biaxial tape-like polymer can also be deduced, assuming that all tapes in solution participate in the network: $h = \phi_p g_N k_B T / G_N^0 l w$, where w is the width of the tape and ϕ_p its volume fraction.

Peptide synthesis and purification. Peptides (K24⁹, Lys β -21¹⁵ and the 11-mer *de novo* peptides) were synthesized and purified according to standard methods. K24 corresponds to residues 41–67 of IsK⁹, with residues 47–49 missing, whilst K27 corresponds to residues 41–67 of IsK.

Phase diagrams. These were constructed from observations of the IR spectra and mechanical properties of solutions of the peptides in a variety of solvents. K24 in HFIP or in the mixtures 50%HFIP/50%CH₂Cl₂ or 90%HFIP/10% methanol forms clear fluid solutions with at least 75% of the peptide in the helical conformation, K24 solutions in solvents such as methanol, 2-chloroethanol, glycerol, ethylene glycol, dimethylformamide (DMF), 70%DMF/30%formamide, DMF/methanol(0–100%), 90%propanol/10%formamide, 50%propanol/50%formamide, water/propanol(30–90%) and HFIP/methanol(70–90%), lie in the gel region (β -sheet conformation $\geq 70\%$). K24 solutions in formamide, water, mixtures of 70%formamide/30%DMF, water/propanol(10–20%), 20%methanol/80%water, 50%HFIP/50%water, and 50% 2,2,2-trifluoroethanol (TFE) 50% water lie above the gel region, whereas solutions in acetone, tetrahydrofuran, diethylether, hexane, dichloromethane, chloroform, 50%methanol/50%dichloromethane, propanol, butanol, hexanol and decanol lie below the gel region. Lys β -21 was studied in similar solvents. In the case of Lys β -21, gelation is achieved initially only at elevated temperatures, depending on the solvent (55 °C for water at pH < 12). This is believed to be governed by the effect of temperature on the aggregation behaviour of the β -tapes. However, the gel, once formed, is stable over the range 10–80 °C, at pH < 12.

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Low-latitude glaciation in the Palaeoproterozoic era

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One of the most fundamental enigmas of the Earth's palaeoclimate concerns the temporal and spatial distributions of Precambrian glaciations. Through four billion years of Precambrian history, unequivocally glacial deposits have been found only in the Palaeoproterozoic and Neoproterozoic record¹. Nonetheless, some of these deposits are closely associated with tropical—rather than just polar—palaeolatitudinal indicators such as carbonate rocks, red beds, and evaporites^{1,2}. These observations are quantitatively supported by palaeomagnetic results indicating a $\sim 5^\circ$ latitude for Neoproterozoic glaciogenic rocks in Australia^{3–5}. Similarly reliable palaeolatitudes for the older, Palaeoproterozoic glaciogenic rocks have not yet been obtained, as such deposits commonly suffer from poor preservation and secondary magnetic overprinting. The Archaean–Palaeoproterozoic 'Transvaal Supergroup' on the Kaapvaal craton in South Africa is, however, exceptionally well preserved, and is thus amenable to the palaeomagnetic determination of depositional palaeolatitudes. Within this supergroup the ~ 2.2 billion-year old Ongeluk lavas are a regionally extensive, largely undeformed and unmetamorphosed, extrusive volcanic succession⁶, which conformably overlies glaciogenic deposits (the Makganyene diamictite). Here we report a palaeomagnetic estimate of $11 \pm 5^\circ$ depositional latitude for the lavas, and hence for the underlying contemporaneous glacial rocks. The palaeoclimate enigma is thus deepened; a largely ice-free Precambrian world was apparently punctuated by two long ice ages, both yielding glacial deposits well within tropical latitudes.

The Ongeluk Formation comprises ~ 500 – $1,000$ m of extrusive, basaltic-andesitic lavas deposited in the marine facies succession of

the Transvaal Supergroup⁷, exposed in the Griqualand West region of the Northern Cape Province (Fig. 1). A correlative volcanic unit, the Hekpoort Formation, occurs in the Chuniespoort region to the northeast (Fig. 1). Isopach trends⁸ indicate that the two volcanic units were deposited in a single, contiguous basin across the Kaapvaal craton⁹. The Ongeluk lavas have been Pb/Pb-dated at $2,222 \pm 13$ Myr old¹⁰, and the Hekpoort Formation has a recalculated Rb-Sr age^{11,12} of $2,177 \pm 21$ Myr. Studies using the U-Pb system in zircon give ages of $2,432 \pm 31$ Myr for the underlying Griquatown Iron Formation¹³ and $\sim 2,050$ Myr for the Bushveld complex¹⁴ which intrudes the Transvaal sequence; these results firmly bracket the age of the Ongeluk-Hekpoort formations between about 2.4 and 2.1 Gyr. The Hekpoort andesites were studied palaeomagnetically by Briden¹⁵, who obtained near-present-field directions by alternating-field demagnetization of samples from only four sites. Of all other penecontemporaneous rocks (2.0–2.4 Gyr) on the Kaapvaal craton, only the Bushveld complex has palaeomagnetic constraints, yielding a range of palaeolatitudes from $\sim 25^\circ$ to $\sim 60^\circ$ (ref. 16).

Palaeomagnetic investigations of other Palaeoproterozoic successions have not conclusively demonstrated primary magnetic remanence. For example, the partly glaciogenic Huronian succession (2.45–2.22 Gyr) in North America has been the object of several palaeomagnetic studies^{17–19}, but those results vary greatly, and a more recent study of the 2.22-Gyr Nipissing diabase and other early Palaeoproterozoic intrusive rocks of the Superior craton casts doubt upon the established apparent polar wander path for the Huronian interval²⁰. Huronian palaeolatitudes thus remain unresolved, partly because of greenschist- or higher-grade regional metamorphism, multiple magnetic components within and among sampling sites, and a lack of unequivocal tests for determining the ages of those components.

In the Northern Cape Province, autochthonous sedimentary rocks of the Transvaal Supergroup are better preserved. Warped into broad, open folds with bedding dips usually less than 5° , the Ongeluk Formation is exposed in several structural troughs in this area. From the mineralogy of the underlying Kuruman Iron Formation, maximum metamorphic temperatures of $<170^\circ\text{C}$ were estimated²¹. All localities discussed herein are road-cut exposures of fresh, fine-grained, massive or pillowed, green-grey lava, except for two sites ("OLL") whose coarse grain size may indicate

that these rocks are intrusive, perhaps feeder dykes for subsequent flows. The remarkably low degree of deformation, low metamorphic grade, and fresh exposures of the Ongeluk lavas provide an ideal opportunity for preservation of primary magnetic directions from the Palaeoproterozoic.

The stratigraphic succession in the Griqualand West region was widely misinterpreted for many years^{6,22} because a large thrust fault was not recognized. In its place, some chose to assign unconformities between virtually all of the formations to account for their juxtapositions; this view has been rejected by subsequent observations and geological syntheses²³ that incorporate the thrust fault and only one major unconformity within the Transvaal sequence, between the Ghaap and Postmasburg groups (Fig. 1).

We present the following field evidence in support of the results outlined by Beukes and Smit²³, that no significant hiatus separates the Ongeluk lavas and the underlying, glaciogenic Makganyene Formation. First, although thicknesses of individual members of the Makganyene Formation vary considerably, this is to be expected in a glacial deposit²⁴, where thickness variations may be due to original facies variability rather than subsequent removal by erosion. Indeed, the lower sections of the Makganyene Formation have widely variable lithology, as seen in boreholes²⁵. Second, the Hekpoort andesite is intercalated with the underlying Boshhoek Formation, partly glaciogenic and correlative with the Makganyene Formation, at several localities²⁶. Third, we and other workers^{25,27} have observed, in borehole cores, apparently conformable contacts with no palaeo-weathering between the diamictite and the Ongeluk lava. Fourth, volcanic shards are abundant within the upper strata of the Makganyene Formation²⁵, indicating that volcanism had already commenced during late-stage glacial times. From the above arguments we contend that no significant plate motion could have occurred between deposition of the diamictite and the lava, and that palaeolatitude estimates of the latter should apply equally to the former.

Visser²⁸ demonstrated a glacial origin for the Makganyene diamictites, citing abundant striated and pockmarked clasts in the Griqualand West region. In addition, during our field work we found cobbles with multiple polished facets and several directions of striations from Makganyene-derived colluvium. Such textures are unlikely to have formed within non-glacial debris flows, but are common in subglacial or glaciomarine environments²⁴. The glacial

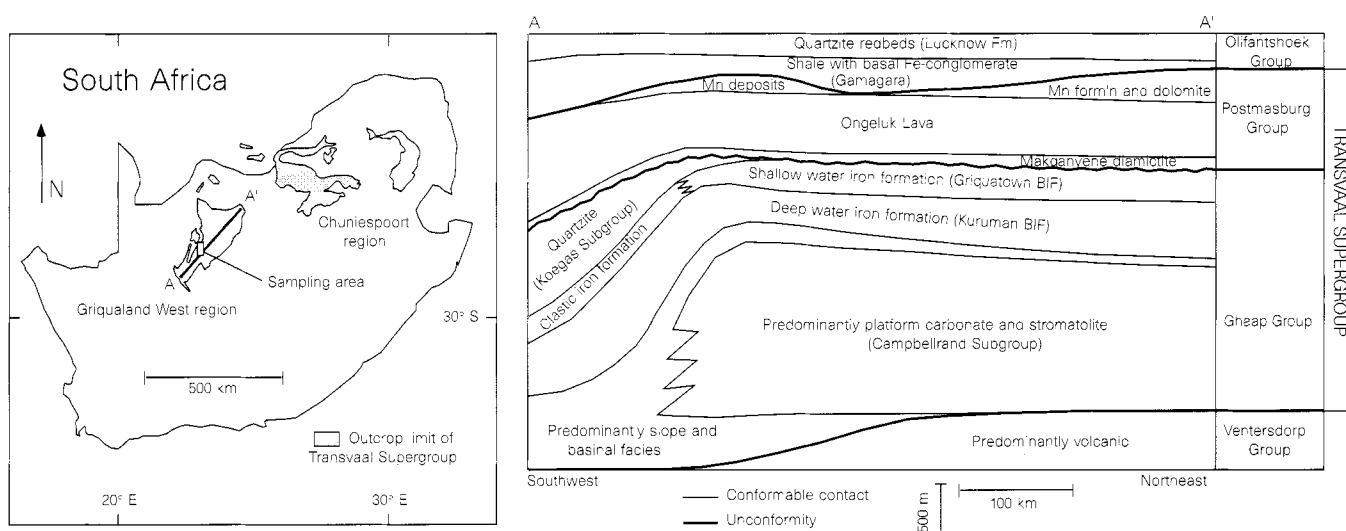


Figure 1 Regional map (left) and schematic stratigraphic overview of the Transvaal Supergroup in the Northern Cape Province⁷ (right). BIF, banded iron formation.

units are fluvial–deltaic throughout the Chuniespoort region, whereas glaciomarine conditions existed in the Griqualand West region⁹. In the latter region, rafted pebbles within mudstone imply a distal environment with input from floating icebergs, and sandstone beds between diamictite bodies are interpreted as subaqueous debris flows²⁵. The sub-Makganyene unconformity involves gentle warping of the lithosphere, less than 1° in the Griqualand West region, which was followed by sheetlike sedimentation over virtually all of the Kaapvaal craton (Fig. 1). Sedimentary indicators all across the craton point to a continental (northeastern or eastern) source for the deposits, ultimately the Limpopo belt or some other subsequently rifted craton⁹; thus the Makganyene Formation cannot be ascribed to local alpine glaciation sourced from either an advancing (offshore to the present west) mountainous terrain or the so-called “Vryburg arch”⁸ which is a post-Transvaal anticlinal flexure now separating the once-contiguous Griqualand West and Chuniespoort ‘sub-basins’ (Fig. 1). The vast regional extent of the Makganyene and equivalent formations in the Chuniespoort region also suggests proximity to a continental ice sheet rather than a local montane source.

Twenty flow-units of the Ongeluk lavas were sampled from 12 localities in the Griqualand West region. Virtually all of the Ongeluk specimens exhibited two magnetic components (Fig. 2). The first to be removed, at the low alternating-field and thermal steps, is directed moderately-to-steeply up and north, coincident with the present local field (PLF) at the sampling sites (Fig. 3a). The coercivity/unblocking spectrum of this component, which is undoubtedly of Recent age, suggests that it is held partly as a viscous-remanent magnetization by multidomain grains, and

partly as a chemical-remanent magnetization by goethite, which is visible along fractured surfaces within a few of the samples.

The higher-coercivity, high-temperature component, observed in 114 of 122 samples of pillow lava or massive andesite, is westerly and of shallow negative (up) inclination (Fig. 3a). Five samples of the coarse-grained rock at a single site (“OLL2”) are oppositely directed; as this site may be intrusive to and slightly younger than the others, its opposite polarity is not surprising. The broad, 30–80 mT coercivity of the westerly component, as well as a narrow ~580 °C unblocking temperature spectrum, suggests that it is carried by single-domain magnetite. Most samples yielded extremely stable, linear demagnetization behaviour (Fig. 2a), conducive to principal-component analysis²⁹. For some of the samples which did not demagnetize to the origin, the trajectories tended towards the present field direction, probably indicating incomplete removal of a haematitic (coercivity >80 mT) component of the Recent chemical-remanent magnetization.

A palaeomagnetic breccia test at one locality (“OVG”) places a constraint on the relative ages of the magnetic components. The hyaloclastic breccia at this site is thought to have formed by explosion of the partly solidified lava on entering shallow water³⁰. The larger clasts have the same megascopic lithology as the overlying and underlying pillowed and massive lavas exposed in the same road-cutting, and in some instances can be identified as fragments of chilled-rim pillows³⁰. Thus, if the large volcanic clasts entrained in the breccia show similar magnetic behaviour to our samples taken from *in situ* flows, then magnetization is primary in the clasts if and only if it is primary in the flows.

As distinct from a conglomerate, whose rounded clasts imply

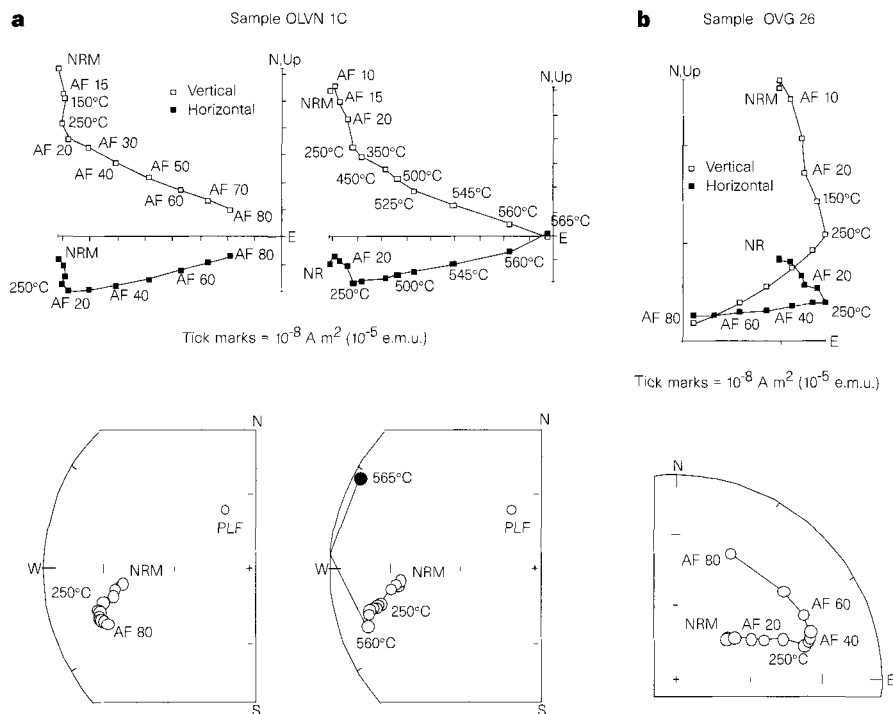


Figure 2 Representative sample demagnetization behaviour. **a**, Sample from *in situ* flow-unit. **b**, Sample from breccia clast. Orthogonal projection diagrams (top): open (solid) symbols depict projection onto the vertical (horizontal) plane. Equal-area projections (bottom): open (solid) symbols show projection onto the upper (lower) hemisphere. AF, alternating-field level in mT; PLF, present local field; NRM, natural remanent magnetization. Samples were either drilled in the field as 2.5-cm cores, or collected as oriented blocks and drilled in the laboratory. Whenever

possible, samples were oriented by both magnetic and solar compasses. Cores were trimmed to 2.2 cm length. Measurement of natural remanent magnetization was followed by alternating-field (AF) and thermal demagnetization, typically measured at the following steps: 5, 10, 15 and 20 mT; 150, 200 and 250 °C; and 30–80 mT with 10 mT intervals. Following the low AF steps, a few samples were thermally demagnetized to 580 or 665 °C; demagnetization was halted when either intensity dropped to zero or spurious magnetizations appeared.

tumbling into truly random orientations, the explosion-induced hyaloclastic breccia is likely to show some differential rotation but not completely random orientations among the clasts. Our breccia test is a statistical modification of the standard palaeomagnetic conglomerate test: instead of testing against a truly uniform distribution, we compare precision of the suite of clasts with the grouping from each of the *in situ* flows. If the precision of directions from a given magnetic component is significantly lower among the clasts than the *in situ* samples, then the test is positive and magnetization of that component probably occurred before brecciation. Alternatively, if the precision among the clasts is similar to precision from each other site, then that component post-dates the brecciation and is not reliable for estimating depositional palaeolatitudes.

Sixteen clasts from the breccia at locality OVG show considerable scatter of characteristic remanent magnetization relative to the massive and pillowed andesite at the same and other localities (see Fig. 3b and Supplementary Information). Individual clasts carry reproducible directions (Fig. 3c), and the suite carries a well grouped PLF overprint similar to that found at other sites (Fig. 3d). Relative precision parameters are scaled against an *F*-ratio distribu-

tion, to determine the likelihood of indistinguishable precision³¹. Common precision between the distribution of each other site and that of the breccia suite can be rejected with >99% confidence for all flow-units, except for site OLL2 (~80%), whose coarse grain size may effect different bulk magnetic properties. The cluster of some of the clast directions towards the southwest may be due to minimal rotation of these clasts during explosive brecciation, or subsequent heating by later flows, or immediate hydrothermal alteration after brecciation. Notably, these samples are from the same proximity in outcrop, with anomalously low magnetic susceptibilities; this may indicate a chemical or mineralogical change due to immediate and localized hydrothermal alteration after emplacement of the breccia¹⁰. Considering the suite of clasts as a whole, however, their wide dispersion rules out any pervasive, secondary remagnetization at locality OVG, and the similarity of directions from *in situ* flows at OVG with other sites supports the interpretation that the characteristic remanent magnetization components were all obtained during deposition of the lavas.

This positive breccia test, in addition to a reversely directed site, implies that the high-coercivity, high-unblocking-temperature, characteristic remanent magnetization component from the *in situ*

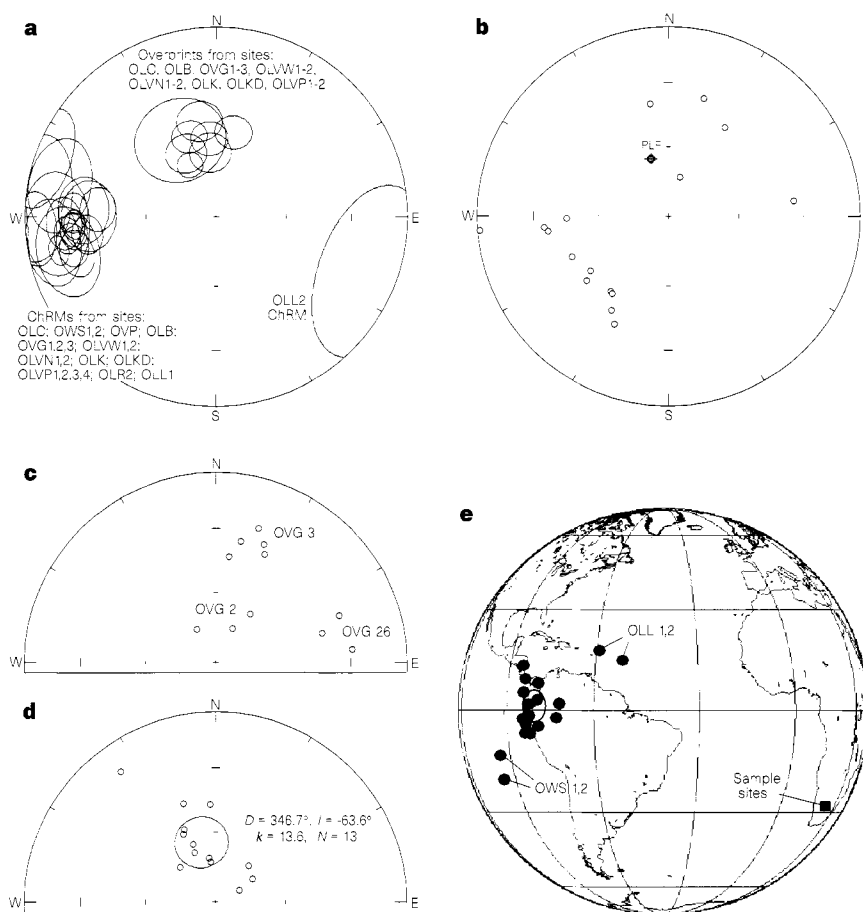


Figure 3 Summary of directional data. Large ellipses are 95% confidence intervals around Fisher mean directions from individual sites (**a-d**), or the mean palaeopole of virtual geomagnetic poles from all sites (**e**). **a-d**, Upper-hemisphere equal-area projections. **a**, Site means of characteristic remanent magnetizations (ChRMs) and overprints. Overprints are coincident with the present local field (PLF) at the sampling sites. **b**, Individual sample ChRMs from the hyaloclastic breccia test, each sample from a distinct clast. **c**, Reproducibility of discordant clast ChRMs. Internal consistency within each clast shows that the large

dispersion in **b** is not spurious. **d**, Individual sample overprints from the breccia test. Good grouping about the present field direction at the sampling sites indicates the expected failure of the breccia test of the Recent overprint, and demonstrates that the wide dispersion in **b** is not caused by errors in sample orientation. *D*, declination; *I*, inclination; *k*, Fisher precision parameter; *N*, number of samples. **e**, Orthographic projection of virtual geomagnetic poles calculated from site-mean ChRMs (pole from site OLL2 inverted).

samples, is probably a thermal-remanent magnetization acquired by initial cooling of the lava flows. Within-flow scatter of directions is small, typically smaller than between-site scatter (see Supplementary Information), and the axially symmetric (Fisherian) distribution of virtual geomagnetic poles (Fig. 3e) supports the model that site-means are thermal-remanent magnetizations of geomagnetic secular variation about an axially geocentric dipole field. We find the tilt-corrected, normal-polarity palaeomagnetic pole at 0.5°N , 280.7°E , implying a depositional palaeolatitude of $11 \pm 5^\circ$ for the Griqualand West region. On the seven-point 'Q' scale³² of reliability, this pole rates a perfect seven.

The low palaeolatitude of the Makganyene diamictite compounds the enigma of Precambrian glaciations. Although glaciogenic deposits of generally Palaeoproterozoic age occur on many cratons, our study provides the first direct and reliable determination of any of these units. It is now documented that both of the broad intervals of Precambrian glaciation, near the beginning and end of the Proterozoic aeon, include glaciogenic sediments deposited in equatorial latitudes. This may indicate a global climate system fundamentally different (due, for example, to changes in Earth's orbital obliquity³³) from that of the past 500 Myr, when glacial deposits were restricted largely to the polar regions. Alternatively, the low-latitude Precambrian glacial deposits could indicate severe, globally inclusive ice ages (a model called the "Snowball Earth"³⁴). In that case, our planet's subsequent recoveries to more mild temperatures would indicate a remarkable resilience to extreme perturbations in climate. □

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Seismic image of the subducted trailing fragments of the Farallon plate

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The Farallon plate was an enormous oceanic plate located west of the Americas during the Cenozoic and Mesozoic eras. This plate has now been almost completely subducted beneath the American plates. In the Northern Hemisphere, the Farallon plate broke up to form a number of independent smaller plates^{1–3} when its western edge approached the North American plate. Here we present a tomographic image of the subducted trailing fragments of the Farallon plate in the upper mantle beneath the western margin of North America. The relatively cold, subducted fragments appear as an intricate region of high seismic S-wave velocity. Comparison of the structure of this high-velocity region with tectonic plate reconstructions and volcanic records enables us to identify individual fragments of the subducted Farallon plate and thus reconstruct qualitatively the kinematic evolution of the Farallon slab in the upper mantle beneath North America.

Relative plate motions of the Pacific, Farallon and North American plates are known from reconstructions based on the magnetic isochrons on the Pacific ocean floor^{4,5}. Estimates of the age of the plate on subduction and of the time passed since its subduction have been used to predict the geometry of the subducted Farallon plate beneath North America over time⁶. In addition, the Late Cretaceous and Tertiary record of arc magmatism^{7–9} in the western United States provides constraints on the slab geometry and its evolution. This record is commonly explained by a flattening Farallon slab and subsequent slab steepening^{2,8,9}. Seismological data can provide important constraints on the slab geometry at depth. Results from early studies and inversions of sparse seismic body-wave travel-time residuals suggest that remnants of the subducted Farallon plate could be located in the upper mantle beneath the western United States^{10,11}. More recently, large suites of body-wave travel times have been used to derive lower-mantle images of older subducted material from the Farallon plate^{12,13}. Here we present our detailed upper-mantle image of the subducted trailing fragments of the Farallon plate.

We applied the method of partitioned waveform inversion^{14,15} to 685 vertical-component broad-band digital seismograms to obtain a three-dimensional image of the S-wave velocity structure of the upper mantle beneath the North American plate. Figure 1 shows the geometry of the wave paths and Fig. 2 shows the resulting three-dimensional image. We suggest that the entire elongated high-velocity anomaly near the western edge of North America (Fig. 2) represents subducted lithosphere from the Farallon plate. The location and coast-parallel trend of the anomaly is consistent with tectonic models of the subducted Farallon plate⁶. The order of magnitude of the S-wave velocity anomaly is also consistent with the expected temperature difference between cold subducted lithosphere and ambient upper mantle, as shown by heat-flow computations for a 60-km thin stalled slab with an initial linear temperature profile from 0 to 1,200 °C (ref. 16). After 26 Myr the temperature anomaly from this slab is still between 200 and 300 °C colder than the surrounding mantle over a region 80 km wide. With an increase

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of $\sim 1\%$ in the S-wave velocity with every 100°C decrease in temperature¹⁷, the imaged high S-wave velocity anomalies can be explained by material that is presently $\sim 200^\circ\text{C}$ colder than the ambient upper mantle, which is consistent with our interpretation of the high-velocity anomalies as subducted oceanic lithosphere from the Farallon plate.

In the Northern Hemisphere, two major fragments of the

Farallon plate have not yet been entirely subducted. The Juan de Fuca plate is currently subducting beneath the northwestern United States and southwestern Canada, while the Cocos plate is subducting beneath Central America. This present-day subduction has been well studied with local seismic data, which has provided detailed images of subducting slab structure^{18,19}. At uppermost mantle depths we also have imaged some high-velocity anomalies related

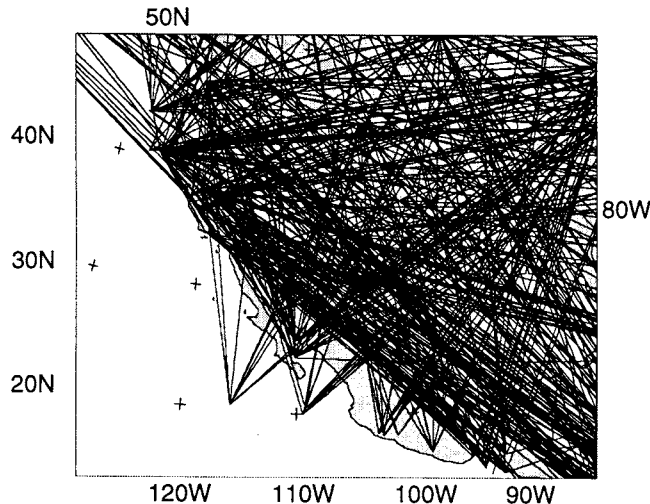


Figure 1 The location, on a map of North America, of the paths travelled by the waves recorded in 685 seismograms. The seismograms are deconvolved with the instrument response to obtain ground displacement records and are windowed around the wave train containing the fundamental and higher-mode Rayleigh waves. The higher modes are sensitive to the structure at deeper levels in the upper mantle, while the fundamental mode is mainly sensitive to the structure of the uppermost mantle. The combination of fundamental and higher modes thus provides good vertical resolution. Good lateral resolution is provided by the large number of wave paths and their wide azimuthal distribution. Synthetic seismograms are computed for a one-dimensional background model that is representative of the average upper mantle between earthquake sources and recording stations. Fine-tuning of the one-dimensional model for each source and receiver pair is performed by fitting the synthetic seismograms to the ground-displacement records with a nonlinear conjugate-gradient optimizer. The resulting path-averaged models are transformed into linear constraints on Earth structure that have uncorrelated errors.

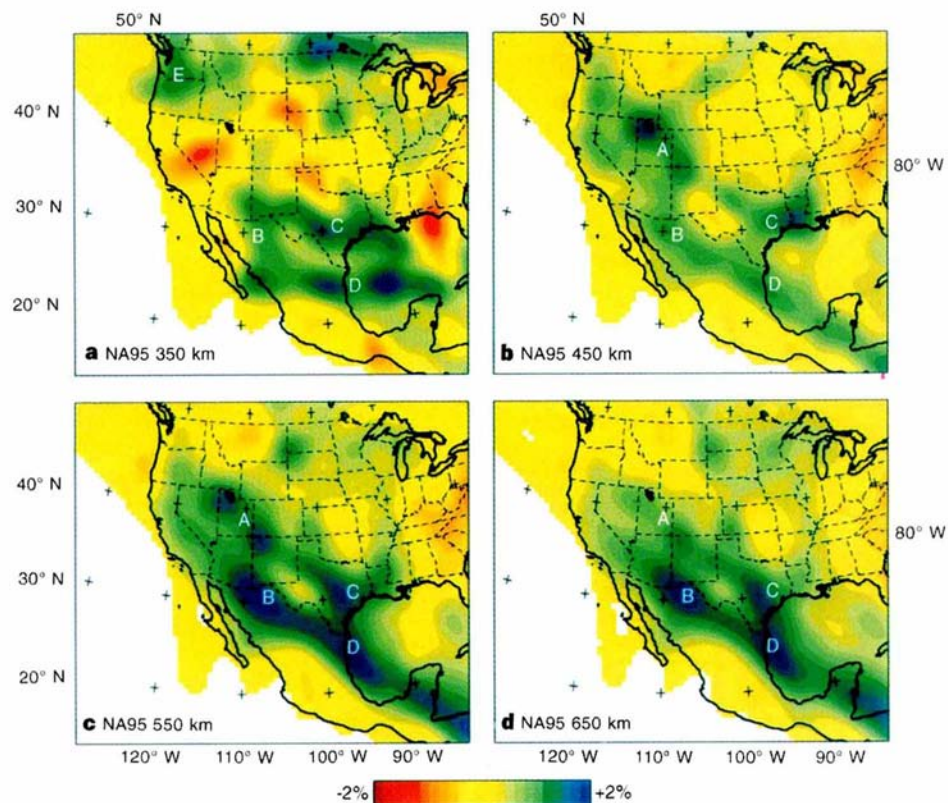


Figure 2 The constraints from all seismograms are combined in a damped linear least squares inversion to yield a three-dimensional model of the S-wave velocity structure of the upper mantle, presented here at different depths, indicated on the maps. Blue and red regions are higher and lower S-wave velocities, respectively. The perturbations are in per cent relative to the background model²⁶ (see colour scale). The model outlines a complex high-velocity anomaly, which we identify as the Farallon anomaly, beneath the western USA and Mexico just above the transition zone (a) and in it (b-d). The high velocities of the Farallon anomaly are labelled with white letters. The image is robust and resolution tests show that,

although the magnitude of the S-velocity anomalies is not well constrained, smooth boundaries of the high-velocity anomaly are spatially resolved to within 200 km (ref. 26). The resolution is best between 15°N and 45°N , although the image is smoothed and the connection between D and B/C may not be real. North of 45°N the resolution degrades at transition-zone depths due to the diminishing sensitivity of the data here. South of 15°N the resolution is less due to degenerating crossing ray coverage (see also Fig. 1). Regions in which the S-wave velocity is not (or poorly) resolved are blanked out.

to the present-day subduction of the Cocos and Juan de Fuca plates, which bracket a region in the uppermost mantle between 18° N and 40° N, without subducted lithosphere. Figure 3 shows a roughly coast-parallel vertical cross-section through our model. In Figs 2 and 3, we have alphabetically labelled the different segments of our image to allow their identification. Based on the positions and trends of the anomalies, we identify anomalies E and D as subducted lithosphere from the subducting Juan de Fuca and the Cocos plates, respectively.

One important feature of our image is the continuous presence, from northwest to southeast, of the Farallon anomaly in the transition zone (Fig. 2b–d). At shallower levels (Fig. 2a) however, anomaly A is missing, thus showing window between anomalies B and C on the southeast side and anomaly E on the northwest side. A window in Farallon subduction started forming 28.5 Myr ago when the Pacific–Farallon ridge first came into contact with North America^{2,6}. Hence we conclude that the material from the Farallon anomaly in the transition zone must have subducted before 28.5 Myr ago. The region of the mantle where subducted lithosphere is no longer present is commonly referred to as the slab window. It has been hypothesized that, in the wake of the downgoing slab, the slab window has been filled in with hot low-viscosity mantle material^{20,21}, generating the Late Oligocene and Miocene record of slab window magmatism^{20,22,23}. This hypothesis is consistent with the presence of low S-wave velocities in the region of the slab window at uppermost mantle depths. The vertical cross-section shown in Fig. 3 runs through the slab window and the underlying Farallon anomaly. Relating progressing geological time to decreasing depth, the cross-section shows the onset of slab window formation at 380 km depth (corresponding to 28.5 Myr ago) and subsequent widening, with progressing time and decreasing depth, of the slab window^{1,2,6,20,21}. The fact that subducted Farallon lithosphere in this region (anomaly A) reached a depth of 380 km in 28.5 Myr or more, provides an upper bound for the vertical sinking rate of this piece of subducted lithosphere of 1.3 cm yr^{-1} .

A second important feature of our image is the intricate structure of the Farallon anomaly. Although anomalies A, B and C all are roughly parallel to the coastline, they are not located at similar distances perpendicular to the coastline (Fig. 2). In particular, while B and C are located behind each other in a coast-perpendicular direction, they are sharply disconnected from A in a coast-parallel direction. Moreover, while B and C form a pair of two individual coast-parallel anomalies, the coast-side boundary of anomaly A seems less clearly defined as the region between A and the coastline is also of somewhat high S-wave velocity (see, for example, Fig. 2c). To identify anomalies A, B and C we have compared their positions to that of North America, in the fixed-hotspot reference frame⁴, during the time of subduction of A, B and C. The position of North America in the period 43–28 Myr ago was several hundreds of kilometers to the northeast of its present-day position⁴. Relative to this position, the boundary between anomalies A and pair B–C is located at the latitude of the present-day northern Gulf of California.

Figure 3 Bottom, vertical cross-section extending from the surface down to the boundary between upper and lower mantle at the 660-km discontinuity. The colour scale is as in Fig. 2. The cross-section is taken along the three combined great circle segments (thick black lines on the accompanying map; top), which follow the trend of the Farallon anomaly.

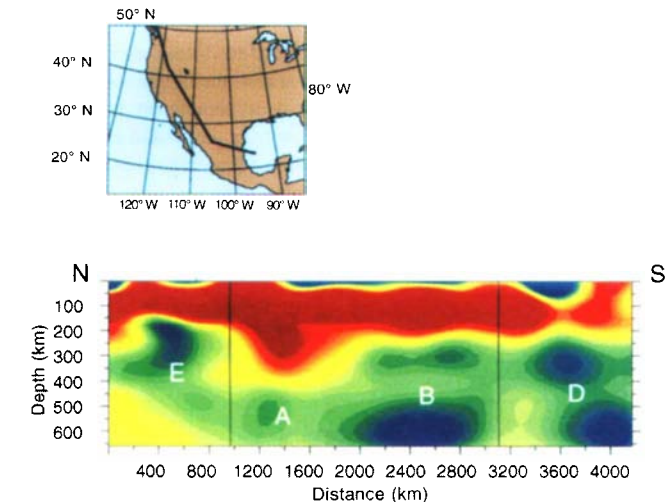


Figure 3 Bottom, vertical cross-section extending from the surface down to the boundary between upper and lower mantle at the 660-km discontinuity. The colour scale is as in Fig. 2. The cross-section is taken along the three combined great circle segments (thick black lines on the accompanying map; top), which follow the trend of the Farallon anomaly.

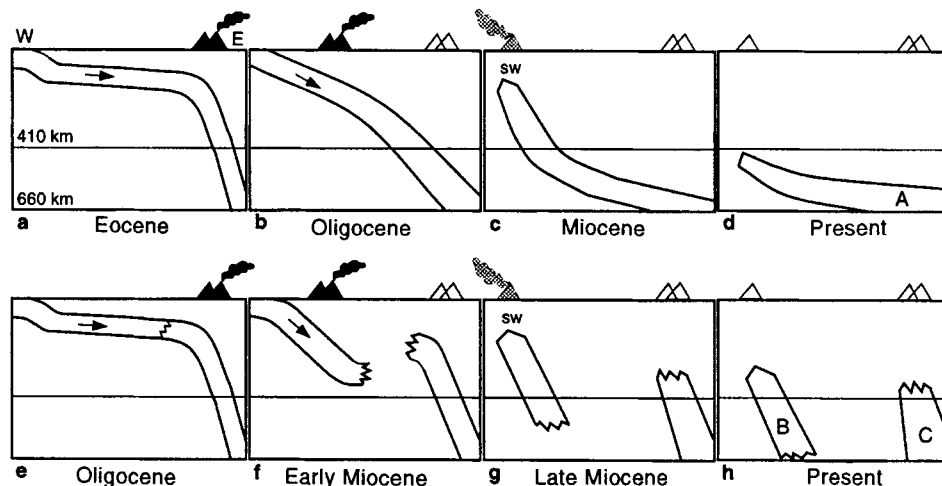


Figure 4 Schematic proposed explanation of the development of anomaly A (a–d) and anomaly pair B and C (e–h). Filled triangles represent active volcanism, open triangles represent inactive volcanoes and the arrows denote the direction of subduction. Both developments start with a subhorizontal subducting Farallon slab, where slab flattening has moved arc volcanism (dark shaded triangles) to the east (a, e). In b, the dip angle of the Farallon slab is increasing, which moves the arc volcanism to the west. Once the western edge of the Farallon plate is subducted (c), the slab window (sw) in the wake of the subducting Farallon slab initiates slab-window magmatism (light shaded triangles). The slab then further

sinks to the transition zone (d), which represents our interpretation of high S-wave velocity anomaly A in Fig. 2. Further south, the flat slab breaks and the dip angle of the near-trench part of the slab increases, moving arc volcanism to the west (f). In g, the two parts of the broken slab continue to sink, the eastern (buoyant) part slower than the western part. In the wake of the subducted western edge of the Farallon plate, the slab window causes magmatism that led to rifting of the Gulf of California. The two parts then sank further into the transition zone (h), which represents our interpretation of high S-wave velocity anomalies B and C in Fig. 2.

This corresponds very well with the position 30 Myr ago of the inferred boundary between two major fragments of the Farallon plate, that is, the Vancouver plate to the north, off which the Juan de Fuca plate evolved, and the remainder of the Farallon plate to the south⁵. The Vancouver–Farallon boundary was located very close and parallel, with a roughly ENE strike, to the Mendocino fracture zone, across which the jump in oceanic plate age was extremely large⁶.

The far northeastward extent of the region that includes the high velocities southwest of anomaly A and anomaly A itself, as well as the far northeastward position of anomaly C, strongly suggest that these anomalies were once related to subduction of Farallon lithosphere at very small dip angles. The volcanic record of western North America indicates that subduction indeed occurred at very small dip angles during the beginning (in the north) and the middle (in the south) of the Tertiary, while corresponding arc magmatism occurred as far east as Colorado^{2,7–9}. Subsequent increase of the slab's dip angle and westward migration of volcanism, also not simultaneous everywhere, was completed around 20 Myr ago^{2,7–9}. A proposed alternative explanation of the volcanic record through downward buckling of the slab²⁴ cannot be reconciled with our tomographic upper-mantle image.

The mechanism of slab steepening beneath continental lithosphere is not well understood. Here we schematically present (Fig. 4) kinematic models for the evolution of the geometry of the Farallon slab beneath western North America, that are consistent with the volcanic record and our tomographic image. To explain anomaly A and the region of somewhat high S-wave velocities to its southwest, we propose a development as schematically presented in Fig. 4a–d. This development for A is consistent with the western United States volcanic record of westward migrating arc volcanism, followed by slab window magmatism^{7–9,20–23}. To explain anomalies B and C we propose a development as schematically presented in Fig. 4e–h. This development for B and C is consistent with the volcanic record^{7–9}, in particular with the migration of Palaeogene arc magmatism in the Sierra Madre Occidental to Neogene arc magmatism in eastern Baja California and coastal Sonora, which shifted into magmatism related to rifting in the Gulf of California²⁵ when all trailing Farallon fragments in this region had completely subducted³. These fragments have since descended some 350 km in about 16 Myr suggesting an average vertical rate of sinking for this piece of subducted lithosphere of about 2 cm yr⁻¹. □

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Hydrostatic locomotion in a limbless tetrapod

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Caecilians are an ancient and enigmatic group of limbless, burrowing amphibians found throughout most of the humid tropics^{1,2}. Over the past 100 million years, the majority of caecilian lineages seem to have retained a series of highly derived musculoskeletal traits from a common ancestor. Among these features are unusually oriented body wall muscles³ and a vertebral column that moves independently of the skin^{4–9}. Until now, these strange characteristics have defied a satisfying functional explanation. Our data suggest that the unique morphology of caecilians enables them to power locomotion hydrostatically by applying force to a crossed-helical array of tendons that surrounds their body cavity. Using this system, the Central American *Dermophis mexicanus* can generate approximately twice the maximum forward force as similar-sized burrowing snakes that rely solely on longitudinally oriented musculature of the body wall and vertebral column for forward force production. Although many groups of invertebrates use hydrostatic systems to move^{10–13} and many vertebrates use hydrostatic systems in localized body parts^{13,14}, caecilians are the first vertebrates known to use the entire body as a hydrostatic system for locomotion.

Because caecilian ribs are not ventrally directed and cannot support their bodies, previous workers have suggested that caecilians maintain body shape during locomotion by means of hydrostatic pressure generated by the unusually vertical muscles of their body wall^{3,4,15,16}. To test this hypothesis, we monitored pleuroperitoneal pressures during vigorous locomotion by implanting air-filled catheters in the vestigial left lung of four specimens of *Dermophis mexicanus* and recording pressure changes with a differential pressure transducer. We expected pleuroperitoneal pressure to vary across different modes of locomotion, but to remain fairly constant within each mode. However, to our surprise, in all four subjects pleuroperitoneal pressure varied cyclically during concertina locomotion. In this mode of locomotion, caecilians use movements

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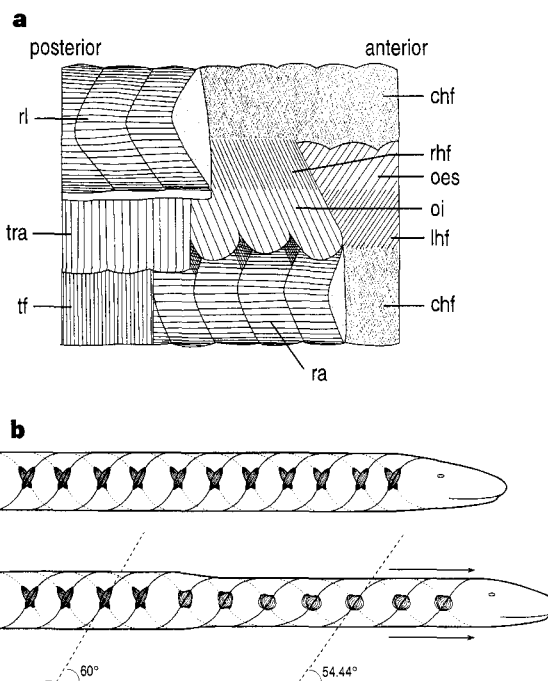


Figure 3 a. Anatomy of the left body wall of *Dermophis mexicanus* from an internal perspective. The layers are depicted from deepest (closest to the viscera) to most superficial (closest to the skin) from left to right. chf, crossed helical fibres; lhf, left-handed helical fibres; oes, m. obliquus externus superficialis; oi, m. obliquus internus; ra, m. rectus abdominus; rhf, right-handed helical fibres; rl, m. rectus lateralis; tf, transverse fibres; tra, m. transversus. **b.** Depiction of current working model of *Dermophis* concertina locomotion and burrowing. The entire helix is placed under tension by internal pressure generated by the transversus muscle. As the obliquus muscles place tension on parts of the helix, it shifts from its resting position of approximately 60° to the long axis of the body, towards 54.44°. As the helix extends, the now rigid outer body wall pushes on the head and aids the vertebral column musculature in generating forward directed force.

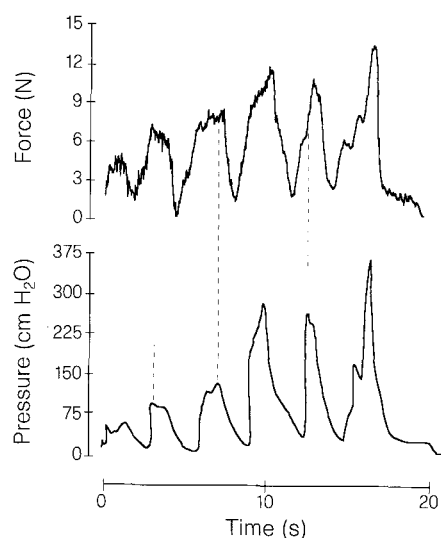


Figure 4 Two *Dermophis* were induced to burrow on the apparatus illustrated in Fig. 2. A fluid-filled catheter was surgically implanted in the pleuroperitoneal cavity so that pressure could be measured simultaneously. The maximum forces achieved by these individuals were less than those achieved before surgery, but a good correlation between pleuroperitoneal pressure and forward force was observed.

that resemble horizontal inchworming to move through straight-sided tunnels. Stationary curves of the body are pressed against the walls of the tunnel to create static points from which the rest of the body can be pushed or pulled¹⁷. In order to determine if the pressure cycles were actively or passively generated, activity of the transversus muscle was also recorded electromyographically. The m. transversus was selected because it was the most easily accessed vertically oriented body-wall muscle and it was the most likely vertical muscle to be universally active during pressure increases. The electromyography revealed that increases in pressure were correlated with forward movement of the head and activity of the transversus muscle (Fig. 1).

In most limbless tetrapods, the longitudinally oriented musculature associated with the vertebral column powers forward movement¹⁸. However, the above observations led us to hypothesize that the vertically oriented muscles of the body wall were helping to power forward movement in *Dermophis* during concertina locomotion. This hypothesis was tested by measuring the maximum forward force the animals could generate and then calculating whether or not the vertebral musculature alone could produce this force. Two individuals were induced to push against a fixed vertical surface from within a channel that was anchored to a force plate (Fig. 2). Under these conditions, both individuals routinely generated 15 N of forward force and sometimes produced 20 N. Given the posture of the animals and the cross-sectional area of the muscles, we calculated that the longitudinal muscles of the vertebral column would have to generate at least 200 N cm^{-2} to account for the measured forces (Fig. 2); this is four times the maximum force per cross-sectional area measured previously in any vertebrate muscle¹⁹. Because the vertebral musculature alone could not produce these forward forces, we concluded that the vertically oriented muscles of the body wall must be assisting the vertebral muscles in powering forward movement.

The vertical muscles of the body wall could produce a forward-directed force by two different mechanisms. In the simpler, the body wall muscles would pressurize the body fluid by reducing body diameter. The pressurized fluid would then push directly on the skull to drive it into the substrate. A second mechanism involves

placing tension on a crossed helix of connective tissue surrounding a fluid-filled tube (in this case the pleuroperitoneal cavity) while the fluid is under pressure. When tension is placed on such a helix, it becomes rigid and changes shape until its constituent fibres are oriented at 54.44° , causing the tube to elongate¹³. In this second system, the rigid outer body wall would push the skull into the substrate. The two obliquely oriented body-wall muscles of *Dermophis* are continuous with a crossed-fibre array of tendons (Fig. 3a)^{3,4} that wind around the body cavity and are oriented approximately 60° to the long axis of the body when the animal is at rest. The completely vertical transversus muscle is in a position to increase body pressure globally (Fig. 3a) so that these obliquely oriented muscles can extend the helix locally (Fig. 3b). Thus, the anatomy of *Dermophis* is consistent with either of the proposed mechanisms.

In order to distinguish between these two mechanisms, we simultaneously measured forward force production and internal pressure in two individuals as they attempted to burrow (Fig. 4). These data allowed us to calculate whether or not the pressures generated in the pleuroperitoneal cavity were high enough to account directly for the forward forces (mechanism 1). The area of the back of the skull in the two *Dermophis* was approximately 1 cm^2 , and the pressures measured would produce only one-tenth of the forces measured on a surface of 1 cm^2 (Fig. 5). Thus, even with the help of the vertebral musculature, this simple mechanism cannot explain the forward forces measured in *Dermophis*. This leaves the second mechanism (extending helix pushing on the skull, Fig. 3b) as the most viable explanation for the data gathered.

The manner in which *Dermophis* changes shape during burrowing offers further evidence for the extending helix hypothesis. During burrowing, the body of *Dermophis* decreases in diameter only in the region between the point from where it pushes and the head. This is consistent with the idea that the helix, already stiffened by an increase in pressure generated throughout the body by the transversus muscle, is being extended locally by the oblique muscles. If global pressure changes were directly pushing on the skull, we would expect uniform changes in body diameter along the entire body.

These data shed new light on the significance of some of the most divergent aspects of caecilian musculoskeletal anatomy. In order for the entire body to function as a hydrostatic system that generates pushing forces, the pleuroperitoneal cavity must be able to change length. This is impossible in most vertebrates, as the tissues that form the body cavity are tightly connected to the vertebral column. However, the specialized trunk anatomy of caecilians, with a loose connection of the skin and associated body wall musculature to the vertebral column, allows them to alter the length of the body cavity. This hydrostatic system appears to be more powerful than a vertebral-driven system, as burrowing snakes of the genera *Farancia*, *Eryx* and *Lococemus* are capable of generating only about 50% of force produced by a *Dermophis* with the same body cross-section (J.C.O'R., N. Kley, D.A.R. and A. P. Summers, unpublished results). In light of these new data, it seems likely that some of the oddest characteristics of caecilian musculoskeletal anatomy have changed little over the past 100 million years because they facilitate exceptional burrowing performance. □

Methods

Concertina locomotion experiments. After anaesthetizing specimens by immersing them in 0.2% methanesulphonate (MS222), an air-filled catheter (Intramedic polyethylene tubing, 1.14 mm internal diameter) was implanted in their vestigial left lung; the catheter was then attached to an Omega PX170-28DV pressure transducer. In two subjects, patch electrodes were implanted on the inner side of the m. transversus. The activity of the other two vertical muscles of the body wall (m. obliquus internus and m. obliquus externus) were not monitored due to the difficulty of securing electrodes to them and small number of individuals available for study. Patch electrodes were constructed

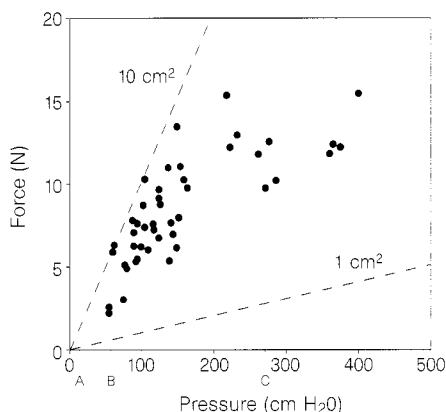


Figure 5 Peak pressure graphed against peak force for 45 thrusts for a single *Dermophis*. Peak pressures from a *Dermophis* at rest (A)⁶, a chuckwalla lizard (*Sauromalus obesus*) defensively inflated in a crack (B)²⁰, and a human performing the Valsalva manoeuvre (C)²¹ are included on the x-axis for reference. The dashed diagonal lines represent the force that would be produced on a given surface area as a function of the pressure on the x-axis using the equation pressure $\times$ area = force.

from Dow Corning Silastic sheeting (500 μm thick) and Teflon-insulated, multistranded, stainless-steel wire (280 μm diameter, California Fine Wire Co.). Individual patches were approximately 4 mm $\times$ 4 mm. The bared portions of the wires were approximately 1 mm long, and the bared wires were separated from each other by approximately 1 mm. Animals were videotaped while performing concertina locomotion in a straight-sided Plexiglas channel. Movement of the head was digitized using Peak Performance 2DTM software and an IBM-compatible computer. Pressure and electromyograms were recorded using Bioware software and an IBM-compatible computer.

Estimation of forward force produced by vertebral musculature. Forward force was measured using a custom-built force plate (Pharos Systems, Inc.) We estimated the maximum forward forces that could be generated by the caecilian's vertebral musculature based on the cross-sectional area of the vertebral musculature and the posture exhibited by the animals as recorded on video tape. The muscles along each side of the vertebral column are never active simultaneously during concertina¹⁸; thus the vertebral muscles act in series and have a maximum physiological cross-section equal to the cross-section of the musculature on one side. The cross-sectional area of one side of the vertebral musculature in the two individuals for which forward force data are available is $\sim 0.25 \text{ cm}^2$. The most liberal estimation of force production by the vertebral musculature assumes that the contraction of the vertebral musculature is isometric, a maximum isometric force production of 50 N per cm^2 of cross-section¹⁹, and an average mechanical advantage of 1. This calculation yields a figure for force production by the vertebral musculature of about 12.5 N. The maximum forward force of 18.7 N measured by us exceeds this by a substantial margin. Furthermore, in reality the vertebral column was bent throughout all burrowing attempts and the mechanical advantage was far less than 1. During sequences in which vertebral posture and forward force were measured simultaneously, the mechanical advantage of the vertebral musculature never exceeded 0.25. On the basis of these more realistic parameters, we estimate that the animals used in this study would have to generate over 400 N of force per cm^2 of muscle cross-section to achieve the maximum measured forward forces if only the vertebral muscles were used.

Calculation of potential force using hydraulic model. In a simple hydraulic system, the amount of force produced by a given pressure will be directly proportional to the area over which the pressure is applied. Applied over a surface area of 1 cm^2 , the approximate surface area of the back of the head of the individuals used, the pleuroperitoneal pressures measured cannot account for the forward forces measured. For example, 100 cm H_2O = 9,785 Pa and 1 $\text{cm}^2 = 1 \times 10^{-4} \text{ m}^2$, thus the amount of force produced by 100 cm of H_2O on 1 $\text{cm}^2 = 9,785 \text{ Pa} \times (1 \times 10^{-4} \text{ m}^2) = 0.98 \text{ N}$. The same pressure would have to be applied over 10 cm^2 to produce the measured pushing forces (Fig. 5).

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Xist has properties of the X-chromosome inactivation centre

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X-chromosome inactivation is the process by which female mammals (with two X chromosomes) achieve expression of X-chromosomal genes equivalent to that of males (one X and one Y chromosome)^{1,2}. This results in the transcriptional silencing of virtually all genes on one of the X chromosomes in female somatic cells. X-chromosome inactivation has been shown to act *in cis* and to initiate and spread from a single site on the X chromosome known as the X-inactivation centre (*Xic*)^{2,3}. The *Xic* has been localized to a 450-kilobase region of the mouse X chromosome⁴. The *Xist* gene also maps to this region and is expressed exclusively from the inactive X chromosome^{3–7}. *Xist* is unusual in that it appears not to code for a protein but produces a nuclear RNA which colocalizes with the inactive X chromosome^{4,8}. The creation of a null allele of *Xist* in embryonic stem cells has demonstrated that this gene is required for X inactivation to occur *in cis*⁹. Here we show that *Xist*, introduced onto an autosome, is sufficient by itself for inactivation *in cis* and that *Xist* RNA becomes localized close to the autosome into which the gene is integrated. In addition, the presence of autosomal *Xist* copies leads to activation of the endogenous *Xist* gene in some cells, suggesting that elements required for some aspects of chromosome counting are contained within the construct. Thus the *Xist* gene exhibits properties of the X-inactivation centre.

The primary role of the X-inactivation centre is to initiate X-chromosome inactivation, leading to a spread of inactivation up and down the X chromosome^{2,3}. In addition, the *Xic* senses whether the cell contains one or more X chromosomes and therefore whether or not it should initiate X inactivation^{10,11}. A 450-kilobase (kb) yeast artificial chromosome (YAC) containing the *Xist* gene has been shown to have the properties of the *Xic*⁴, but the possibility was left open that multiple genes in the region are required for a functional *Xic*. To determine whether the *Xist* gene alone is sufficient to mediate *cis* inactivation and counting, we devised an assay using embryonic stem (ES) cells. Female ES cells have two active X chromosomes and upon differentiation *in vitro* into embryoid bodies (EBs), one of the X chromosomes becomes inactivated¹⁰, probably because of the presence of two *Xics*^{10,11}. Furthermore, males carrying two X chromosomes undergo X inactivation. We reasoned that, if *Xist* could function as an *Xic*, the introduction of an additional *Xist* gene into male ES cells might lead to the inactivation of a linked reporter gene upon differentiation.

Using homologous recombination in yeast, we generated a

Table 1 Activation of the endogenous *Xist* gene in male ES cells by the presence of ectopic copies of *Xist*

Cell line	Subclones analysed	Cosmid <i>Xist</i> derived	ES cell <i>Xist</i> derived	ES cell <i>Xist</i> (%)
ZH β 10	27	19	8	29.6
ZH β 2	17	12	5	29.4

Analysis of expression from the endogenous *Xist* gene. RNA prepared from EBs of clones ZH β 10 and ZH β 2 was reverse-transcribed into cDNA and *Xist* sequences amplified by PCR. The PCR products were subcloned into pBluescript and individual clones were sequenced and assigned as being derived from RNA originating from either the cosmid or the endogenous *Xist* gene.

specific cosmid construct containing the *Xist* gene with 9 kb of 5'- and 6 kb 3'-flanking sequences, and β GEO (a β -galactosidase-neomycin-resistance fusion gene) driven by the HMG-CoA reductase (*HMG*) promoter¹² (Fig. 1). The *HMG* promoter can respond to inactivation when located on the X chromosome¹³. Furthermore it is not spontaneously extinguished upon cell differentiation, unlike the commonly used PGK promoter (L.H. and J.H., unpublished results, and ref. 14). The cosmid was linearized with lambda terminase and transfected into male (XY) CCE ES cells.

We derived ES-cell transfectants that were positive for β -galactosidase (β -Gal) and resistant to G418 and contained the cosmid or, as controls, the *HMG*- β GEO vector or a 20-kb vector containing all the cosmid vector sequences but only 3 kb of 3' *Xist* gene sequence. These transfectants were allowed to differentiate into EBs, which were cultured in suspension in non-selective medium for 4–5 days; individual EBs were then plated and differentiated cells were allowed to grow out. Terminally differentiated cells in each EB were visually scored for β -Gal activity following X-Gal staining 5–10 days after plating. Characteristic staining patterns before and after differentiation are shown in Fig. 2A and a time course of β -Gal activity in Fig. 2B. Most EBs derived from cosmid transfectants contained few β -Gal-positive cells in the differentiated outgrowth (Fig. 2A, a and b), whereas β -Gal activity was retained in most of the differentiated cells in the control vector *HMG*- β GEO transfectants (Fig. 2A, c and d), as well as in transfectants carrying the control 20-kb plasmid (data not shown). This latter control rules out the possibility that the bacterial vector sequences are influencing expression of the marker gene. In addition, *Xist* cosmid transfectant EBs exhibit minimal outgrowth in the presence of G418 (<15% EBs) and G418 addition after differentiation kills established outgrowth. Surviving ES-like cells were strongly β -Gal-positive. These results confirm that the β -Gal-neo fusion gene is downregulated even in the presence of G418 when there is selection for gene activity. Control transfectant EBs, on the other hand, are not similarly downregulated and show frequent (>50% EBs) and substantial β -Gal-positive outgrowth in the presence of G418 (data not shown).

To investigate the mechanism of downregulation of β -Gal activity, we prepared RNA from selected clones and examined the expression of the *Xist* and β -Gal genes by northern blotting (Fig. 3). The 15-kb *Xist* RNA was strongly induced upon differentiation. The presence of *Xist* RNA in ES cell cultures has been described previously and is probably due to a small proportion of spontaneously differentiating cells¹⁵. The induction of *Xist* RNA shows that the 9 kb of 5' and 6 kb of 3' DNA flanking the *Xist* gene are sufficient to recapitulate the normal expression pattern of the gene upon ES-cell differentiation. The induction of *Xist* RNA coincides with downregulation of β -Gal RNA (Fig. 3), suggesting that β -Gal transcription is being switched off as a result of *cis*-inactivation. The absolute levels of messenger RNA for both *Xist* and β -Gal varied between transformants and appeared not to depend on copy number; this is to be expected, given the small amounts of flanking

sequence introduced¹⁶ and could reflect a requirement for additional regulatory sequences.

The mechanism whereby a cell counts the number of X chromosomes and hence knows whether to initiate the process of X inactivation is unknown, but may depend on the ratio of the number of X chromosomes to the number of autosome sets^{9,10}. Sensing that there is more than one X chromosome and the consequent initiation of X inactivation is thought to be a property of *Xic*. However, it is possible that the two activities are separable and that our construct is sufficient for *Xist* activation upon ES-cell differentiation but lacks the sequences required for chromosome counting. Alternatively, if the construct contained counting elements (and so was equivalent to *Xic*), we might expect some of the cells carrying the cosmid transgene to activate the cosmid *Xist*

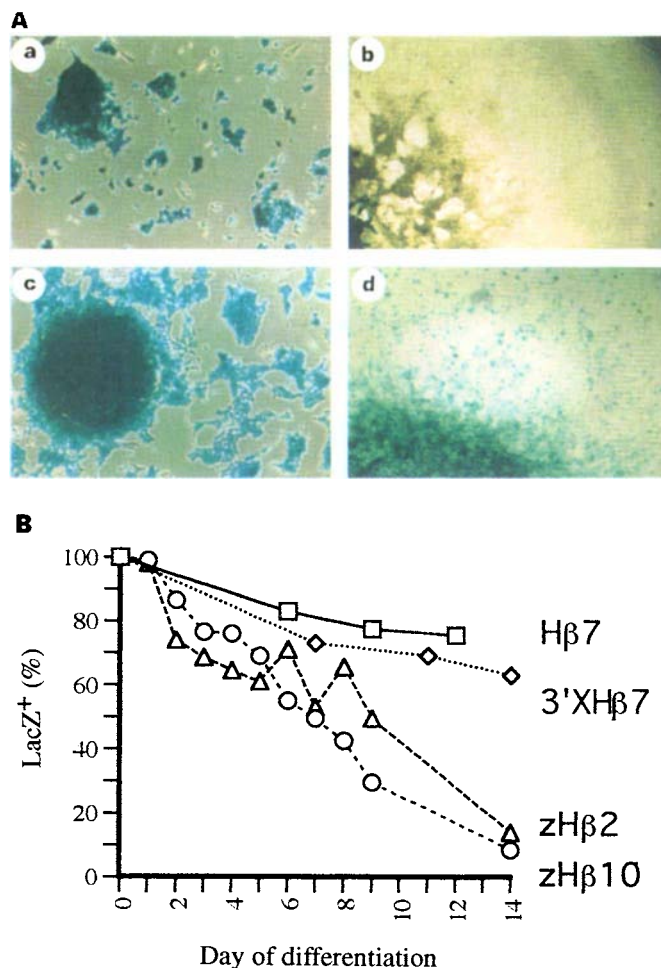


Figure 2 A Reporter gene linked to *Xist* is inactivated on ES-cell differentiation.

A, Representative X-Gal-stained ES-cell transfectants before and after differentiation. **a, b**, *Xist* cosmid transfectant zH β 10; LacZ-positive ES cells (**a**) and section of EB showing differentiated outgrowth (**b**). ES cells from the centre of the EB (lower left-hand corner) retain LacZ staining, which is lost in almost all differentiated cell outgrowth. **c, d**, *HMG*- β GEO control transfectant H β 2; LacZ-positive ES cells (**c**) and section of EB showing minimal loss of LacZ staining upon cell outgrowth and differentiation (**d**). **B**, Loss of β -galactosidase expression in representative cosmid transformants compared with controls during differentiation: Samples of differentiating EBs grown in suspension were taken at 24-h intervals and stained with X-Gal. Total LacZ activity was determined by averaging the percentage of LacZ-positive cells/EB for 75–100 EBs at each time point. zH β 2 and zH β 10 are ES cell lines carrying the *Xist*/reporter cosmid construct. Controls shown are H β 7 (the *HMG*- β GEO vector) and 3'XH β 7 (a 20-kb vector containing all the cosmid vector sequences but only 3 kb of 3' *Xist* gene sequence).

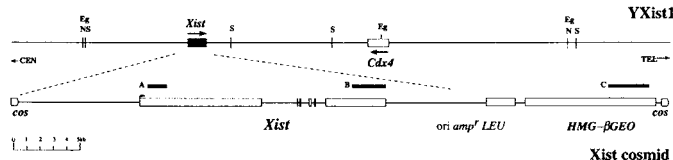


Figure 1 Structure of the *Xist* cosmid reporter construct¹². The YAC YXist1 is shown above the cosmid construct derived from it by homologous recombination in yeast. Probes used to determine fidelity and copy number of cosmid integrants into ES cells are indicated as bars labelled A–C. The *Leu* gene is a selectable marker used for construction of the cosmid in yeast¹². The linearized cosmid contains partial *cos* sequences at each terminus. Selected restriction enzyme sites are indicated: Eg, *Eag*I; N, *Not*I; S, *Sal*I.

(inactivating the autosome) and some cells to activate the endogenous *Xist* (inactivating the single X chromosome). We therefore developed an assay based on reverse transcription and the polymerase chain reaction (RT-PCR), using a sequence polymorphism¹⁷ between the mouse strains C57BL/10 (from which the cosmid are derived) and 129 (the strain from which the ES cells are derived) to determine whether the endogenous *Xist* gene is appreciably expressed. This assay showed that ~30% of the *Xist* RNA in two cell lines (zH β 10 and zH β 2) at the time assayed (8 days post-differentiation) was derived from the endogenous (ES cell) *Xist* gene (Table 1). *Xist* RNA in male ES cell cultures and EBs is normally present only in extremely small amounts^{4,6,15} and so the presence of ectopic *Xist* copies must have led to the activation of the endogenous gene in some cells. RNA/DNA fluorescence *in situ* hybridization

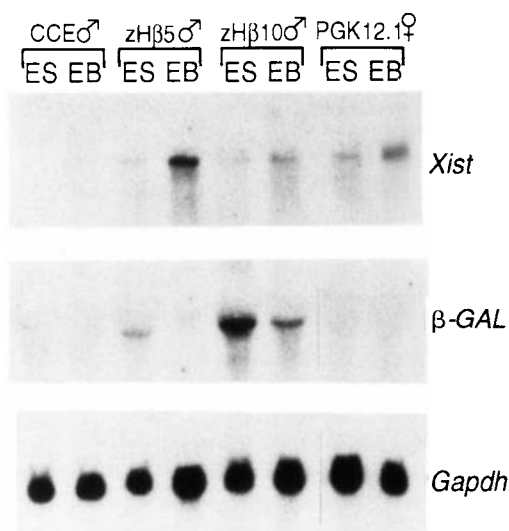


Figure 3 Activation of *Xist* gene expression on differentiation of ES-cell cosmid transfectants. RNA was prepared from ES cell and EB cultures, electrophoresed on an agarose/formaldehyde gel and blotted onto a nylon membrane. This was hybridized with probes for *Xist*, β -Gal and, as a control, *Gapdh*. ES cell lines CCE (male) and PGK 12.1 (female), as well as two cosmid transfectants zH β 5 (2 copies) and zH β 10 (8 copies) are shown. Another line, zH β 2 (6 copies), also showed activation of *Xist* RNA and repression of β -Gal RNA on differentiation, although the *Xist* RNA was induced to a lesser extent (see Supplementary Information).

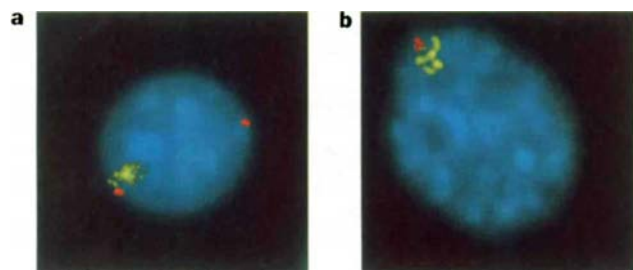


Figure 4 *Xist* RNA is located close to the autosome carrying the cosmid transgene. **a**, RNA/DNA FISH on a control female cell (c127 fibroblast cell line¹⁸). The *Xist* RNA cluster (green) is localized to one of the X chromosomes (red). The *Xist* RNA probe was the biotin-labelled PCR product of *Xist* exons 1 and 6. X chromosome localization was determined using a DIG-labelled P1 clone which maps to the telomere of the X chromosome. **b**, RNA/DNA FISH on cosmid transfectant zH β 10. The *Xist* RNA cluster (green) localized to the autosome carrying the cosmid (red) in almost all cells that gave both signals. Integrated cosmid was detected using DIG-labelled HMG- β GEO plasmid. Further material is available as Supplementary Information.

(FISH) analysis supports this idea (see Supplementary Information). Our lines contain multiple (between 2 and 8) copies of the construct located at single independent sites and we do not know if all the copies are active or counted individually as *Xics*. Hence we cannot assess whether all aspects of X-chromosome counting are operating correctly. However, activation of endogenous *Xist* on differentiation makes it highly likely that the basic counting mechanism has been activated by the introduction of ectopic *Xist* genes and that the cells are behaving as if more than one X chromosome (*Xic*) is present in the cell. Such *trans* regulation of an endogenous gene by introduced copies is unusual in vertebrates and can now be investigated in our system. *Xist* expression from the endogenous X chromosome probably leads to X inactivation, which would be lethal in male cells; this has been observed in male ES cells carrying ectopic copies of YACs carrying *Xic*⁴. Our finding that cell death increases on differentiation of cosmid transfectants is consistent with this induced and aberrant X inactivation (see Supplementary Information).

RNA/DNA FISH studies have shown that *Xist* RNA colocalizes with the inactive X chromosome at interphase, perhaps by interaction with the nuclear matrix^{4,8}. We have used similar techniques to investigate the subnuclear location of *Xist* RNA in the cosmid transfectants (Fig. 4). In control female cells (c127 mouse fibroblast cells¹⁸), a punctate cluster of *Xist* RNA signal, similar to that previously described^{4,8}, was found close to one of the X chromosomes (presumably the inactive X). In the *Xist* cosmid transfectant shown in Fig. 4, the cluster of *Xist* RNA signal is close to the cosmid integration site (detected with a HMG- β GEO probe) in most of the cells. The mechanism of *Xist* RNA localization to the inactive X chromosome is not fully understood but may involve interaction with the nuclear matrix surrounding the chromosome rather than on-going transcription or passive localization following transcription⁸. Whatever the mechanism, our results exclude the possibility of the X chromosome or its nuclear domain having unique properties that allow it to associate with *Xist* RNA.

The main features of the *Xic* are to recognize the number of X chromosomes (>1) and to act as the point of origin for inactivation. Previous studies, using an *Xist*-deletion mutant, showed that *Xist* is necessary for *cis* inactivation but left open the possibility that other genes might be involved and did not define the sequences required for counting⁹. YAC transgenesis localized *Xic* function to a 450-kb region of the X chromosome that contains several genes, including *Xist*⁴. Our results indicate that *Xist* can act as a countable element to recapitulate at least some aspects of chromosome counting and that it is sufficient to produce gene inactivation *in cis*. Thus *Xic* is most likely to be composed of the *Xist* gene alone. The mechanism of inactivation itself remains to be determined but may well involve the specific localization of *Xist* RNA that we observe. In addition to providing insight into the molecular basis of X inactivation, the ability of the *Xist* gene to cause inactivation *in cis* may be a useful complement to deletion analysis¹⁹ in identifying recessive mutants and haploinsufficient or imprinted regions of mammalian genomes. □

Methods

Construction of *Xist*/reporter cosmid and generation of ES-cell transfectants. The *Xist* gene was recovered from the YAC YXist1 and incorporated into a cosmid construct together with the HMG- β GEO gene as described¹². Briefly, homologous recombination in yeast was used to target phage λ *cos* sites flanking the *Xist* gene. The HMG-CoA reductase gene promoter²⁰ linked to a neomycin-resistance/ β -galactosidase fusion gene (β GEO²¹) was incorporated as a reporter gene. Cosmids were rescued using lambda-packaging extract and bacterial transduction. The resultant cosmids were linearized by treatment with λ terminase, and DNA was introduced into male CCE ES cells (from T. Enver) using lipofectamine (Gibco). ES cells were grown on mitomycin C-treated feeder cells and transfectants selected in G418.

Copy number of transfectants was determined by Southern blotting using a probe in *Xist* exon 6 (probe B in Fig. 1) which differentiates between exogenous *Xist* sequence and the transgene by a unique *EcoRI* restriction site in the cosmid. PhosphorImager analysis indicated that cosmid copy numbers were 6, 2, and 8 for transfectants zH β 2, zH β 5, and zH β 10, respectively. Further analysis using probes for 5' *Xist* exon 1 or β -Gal (probes A, C in Fig. 1) indicated that the cosmid integrants were probably intact. FISH analysis of zH β 2 and zH β 10 confirmed that integrants were at single distinct autosomal sites in the genome.

LacZ-positive, G418-resistant ES cell transfectants were differentiated by EB formation as follows: semiconfluent ES cell populations were lightly trypsinized and cell clusters were cultured in suspension on bacterial dishes in DMEM medium supplemented with 10% FCS, without LIF feeder cells or G418, for 4–5 days. To analyse LacZ-staining activity and sensitivity to G418 upon differentiation, individual EBs were aspirated and plated into gelatinized 96-well dishes and allowed to adhere. 1–3 days later, half of the adhered blasts were transferred to medium containing G418, and 5–10 days later stained for LacZ activity using X-Gal²². For temporal analysis of LacZ staining during differentiation, EB samples were collected from suspension cultures and stained with X-Gal at 24-h intervals.

RNA analysis. ES cells were prepared for RNA isolation by multiple passages onto gelatinized dishes without feeder cells to prevent contamination by feeder-derived RNA. EBs were cultured in suspension for 8–10 days and collected for RNA preparation when the majority of EBs had become cystic. RNA was isolated using RNazol B (Biogenesis Ltd) according to the manufacturer's instructions. 10 μ g of each RNA sample was electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto Hybond N. The *Xist* probe was a 9.5-kb PCR fragment derived from exon 1 of the *Xist* gene. β -Gal RNA was detected with probe C (Fig. 1). Cosmid *Xist* (C57BL/10-strain-derived) and CCE ES cell *Xist* (129-strain-derived) were distinguished by a single base difference⁷. As the polymorphism is within a poly(A) tract, it is not amenable to direct analysis. cDNA flanking the polymorphism was amplified by RT-PCR (primers were 5'-ACGCGTCGACGTGTGTATGGTGG ACTTACC-3' and 5'-CCATCGATATCAGCAGCAACAGTACAG-3') from RNA prepared from the transfectants and subcloned into pBluescript. Individual clones were randomly sequenced and assigned according to their RNA derivation from the introduced cosmid *Xist* gene or from the endogenous CCE *Xist* gene.

DNA and RNA FISH. Metaphase spreads were obtained from ES cells and FISH was performed as described⁸. 100–200 ng biotinylated probe was used for hybridization, together with 2 μ g mouse COT-1 DNA and 5 μ g salmon sperm DNA. Slides were incubated overnight at 37°C and washed stringently. Probe was detected using two rounds of FITC-conjugated avidin amplification. FISH signal detection was performed using a Zeiss Axioscop microscope equipped with epifluorescence and a triple-band-pass filter by analysis of digital images from a high-resolution CCD camera (Photometrics, USA) and imaging software (Digital Scientific). ES and differentiated cells were prepared for DNA/RNA FISH by modification of standard techniques⁸: ES cells were grown on gelatinized slides, fixed by air-drying, permeabilized with 0.5% Triton-X 100 in CSK (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES, pH 6.8) buffer and fixed with cold 4% paraformaldehyde. Slides were stored at 4°C in 70% ethanol. Cell populations were enriched for differentiated cells by plating EBs grown without G418 selection in suspension for 4 days onto gelatinized dishes. The mixed population was then trypsinized and allowed to adhere to non-gelatinized tissue-culture dishes for 1 h, when non-adherent (ES) cells were aspirated and fresh medium added. This procedure was repeated following growth of plated cells for 2–3 days; however, cells were allowed to adhere to gelatinized glass slides. Slides containing differentiated cells were fixed and stored as described. Slides prepared for DNA/RNA FISH (Fig. 4) were dehydrated through an ethanol series to 100% ethanol, dried under vacuum, and hybridized overnight at 37°C. Slides were washed and the biotin signal detected as described; as a control, some slides were treated with RNaseH before signal detection and fixation. The RNA signal was fixed for 5 min in 4% paraformaldehyde; slides were then dehydrated, denatured, dehydrated again and hybridized to a DIG-labelled DNA probe. The DNA signal was detected following a medium-stringency wash with Texas red-conjugated anti-sheep or rhodamine-conjugated anti-DIG antibodies. The *Xist* RNA probe was the biotin-labelled PCR product of *Xist* exons 1 and 6. X chromosome localization was achieved using a biotin- or biotin/DIG-labelled P1 clone (ICRFP703D1773;

ref. 23) which maps to the telomeric region of the X chromosome (L.H., unpublished result).

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Long-range *cis* effects of ectopic X-inactivation centres on a mouse autosome

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In mammals, the X chromosome is unique in being capable of complete inactivation. Such X inactivation evolved to compensate for gene dosage differences between females with two X chromosomes and males with one¹. Transcriptional silencing of a single female X chromosome is controlled *in cis* by *Xist*², whose RNA product coats the inactive X chromosome (X_i)³, and the X-inactivation centre (Xic)⁴. A transgenic study limited the *Xic* to 450 kilobases including *Xist*, and demonstrated that it is sufficient

to initiate X inactivation⁵. Here we report that ectopic *Xist* RNA completely coats transgenic chromosome 12. Expression of genes over 50 centimorgans was reduced two-fold and was detected only from the normal homologue in fibroblasts. Moreover, ectopic *Xic* action resulted in chromosome-wide changes that are characteristic of the X_i : DNA replication was delayed, and histone H4 was markedly hypoacetylated. Our findings suggest long-range *cis* effects on the autosome similar to those of X inactivation, and imply that the *Xic* can both initiate X inactivation and drive heterochromatin formation. Thus, the potential for chromosome-wide gene regulation is not intrinsic to X-chromosome DNA, but can also occur on autosomes possessing the *Xic*.

X inactivation consists of several steps, including the counting of X chromosomes^{2,6}, commitment of all but one of these to inactivation¹, initiation at *Xic*^{4,6}, induction of *Xist* expression⁷⁻⁹, propagation of *Xist* RNA along the X chromosome³, and establishment and maintenance of heterochromatin throughout the X chromosome¹⁰. We had previously generated transgenic murine embryonic stem (ES) cell and fibroblast lines in which the *Xic* carried on the yeast artificial chromosome Y116 was transplanted onto autosomes in male cells⁵. We found that the 450-kb transgene recapitulated steps leading up to *Xist* expression and spread of *Xist* RNA into adjacent autosomal DNA. Here we investigated whether events initiated by ectopic *Xics* can be completed on autosomes, and whether inactivation can occur independently of context or whether establishment and maintenance of heterochromatin require additional *cis*-acting elements specifically along the X chromosome.

To test whether the potential for large-scale inactivation lies in intrinsic differences between X and autosomal DNA, we examined *cis* effects of ectopic *Xics* on the autosome in the male transgenic fibroblast line 116.6 (ref. 5). This line was isolated from mouse chimaeras derived from male ES cells carrying >20 tandem copies

of Y116 in a 40XY background. However, because simian virus 40 (SV40) large T transformation was necessary for fibroblast cloning, each fibroblast line contains diploid (<30%) and tetraploid (>70%) cells. Like diploids, tetraploids inactivate all but one X chromosome per diploid genome. To identify the autosome of transgene insertion, we performed fluorescence *in situ* hybridization (FISH) using mouse chromosome-specific paints and the Y116 probe (Fig. 1a, b). Co-localization of Y116 signals and autosomal paints revealed a pericentromeric integration into chromosome 12 (Ch12).

To address whether *Xist* RNA can coat autosomes as it does the X_i , we examined interphase and metaphase chromosomes using two-colour FISH which simultaneously detected *Xist* RNA and Ch12. Over 95% of metaphases ($n > 50$) from the 116.6 fibroblasts demonstrated spread of *Xist* RNA throughout transgenic Ch12 (Ch12.Tg; Fig. 1c, d). Apart from the centromere, there were no apparent skip regions. The RNA did not bind other chromosomes, including the X. Association was also observed during interphase when *Xist* RNA localized to the Ch12 domain in cells prepared using two fixation techniques (Fig. 1e, f). Thus *Xist* RNA can associate with the autosome as it does with the X_i , and exhibits the same *cis*-restriction at the ectopic locus. In contrast to human *XIST* RNA³, murine *Xist* associates stably with both X_i (B. Panning, unpublished data) and Ch12 during interphase and mitosis until late metaphase.

The genetic distance of Ch12 is 60–70 cM (ref. 11). To examine effects on transcription, we assayed expression of Ch12-linked housekeeping genes at various distances (Fig. 2a)¹¹: *Rrm2* (ribonucleotide reductase M2) at 7 cM from the centromere, *mSos2* at 30 cM, *c-Fos* at 40 cM, and *Yy1* at 53 cM. We used quantitative reverse transcription–polymerase chain reaction (RT–PCR)¹² to investigate whether Ch12 gene expression is reduced in transgenic fibroblasts compared with control isogenic fibroblasts. Quantitization was made possible by amplifying in the exponential phase and normalizing to expression of *Dnmt*, a housekeeping gene on Ch9. Expression of *Rrm2* and *mSos2* was twofold lower in transgenic ($n = 8$) than in control mice ($n = 8$) (Mann–Whitney test, $P < 0.001$; Fig. 2b, c, e). Importantly, these differences did not result from aneuploidies, as Ch9 and Ch12 were present at two copies per diploid genome (four in tetraploids) in transgenic and control mice, as demonstrated by chromosome painting (data not shown). In contrast, there was no reduction ($P > 0.13$; Fig. 2d, e) for the unlinked gene ribosomal protein L7 (*RpL7*) residing on Ch2 (also not aneuploid).

We used RNA FISH to visualize nascent *Yy1* and *c-Fos* expression. As *c-Fos* is induced by serum, control and transgenic fibroblasts were grown for 24 h in serum-free DME, then induced by 20% fetal bovine serum/DME, and paraformaldehyde-fixed after 15 min (ref. 13). RNA FISH was performed without DNA denaturation to exclude DNA detection. The authenticity of RNA signals was established by subsequent hybridization to Ch12 paint or to the subtelomeric marker D12mit196 (data not shown). In control female cells, *c-Fos* RNA was visible in 40% of serum-induced cells and in 5% of uninduced cells, consistent with previous observation¹³. *Yy1* RNA could be seen in 30% of cells regardless of whether induction had occurred. To determine the origin of *c-Fos* and *Yy1* transcription in 116.6 fibroblasts, nascent RNA signals were scored for co-localization to *Xist* RNA, which marks the Ch12.Tg domain. Three patterns of expression were found (Fig. 3): (1) nascent RNAs not overlapping with *Xist* RNA (scored as monoallelic from wild-type Ch12); (2) nascent RNAs originating from within *Xist* RNA domains and from external loci (scored as biallelic); and (3) no detectable nascent RNA. In tetraploids, a signal from one or both copies of Ch12 or Ch12.Tg was scored as expression. In controls expressing *c-Fos*, the predominant pattern was biallelic (67 of 70); in transgenic cells, the predominant pattern was monoallelic (57 of 61; Fig. 3a, c). Similarly, *Yy1* was predominantly biallelic in controls (33 of 40) but mostly monoallelic

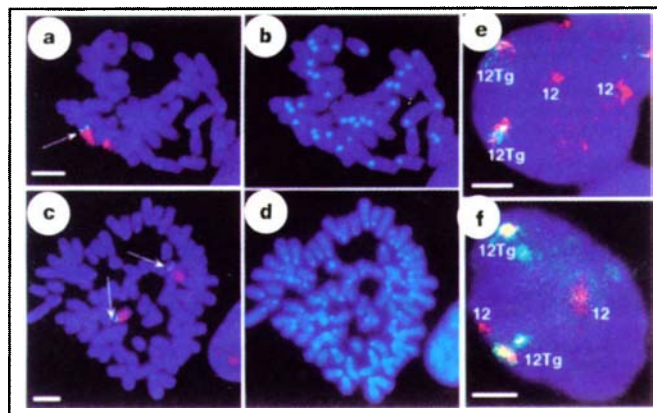


Figure 1 *Xist* RNA completely coats mouse Ch12. **a, b**, Mapping of transgene insertion site in ES line 116.6 by two-colour FISH (**a**) using FITC-labelled Y116 (green) and Texas red-labelled Ch12-specific paint (red), demonstrating pericentromeric insertion of the 20-copy transgene (arrow). The single-copy endogenous *Xic* is not evident because of short exposure time. Chromosomes were counterstained by DAPI (**b**). **c, d**, *Xist* RNA coats all of Ch12.Tg in metaphase ($n > 50$; note tetraploidy). To visualize *Xist* RNA, undenatured chromosomes were hybridized to rhodamine-labelled *Xist* probe. To detect Ch12.Tg, subsequently denatured chromosomes were probed with FITC-labelled Y116 (arrows). The two images are superimposed in **c**. Chromosomes were counterstained with DAPI (**d**). **e, f**, Localization of *Xist* RNA to the Ch12 domain in interphase nuclei ($n > 100$). Cytogenetically prepared cells (**e**) and cells grown *in situ* (**f**) were hybridized to FITC-labelled *Xist* probe, and subsequently denatured for hybridization to Texas-red conjugated Ch12-paint. Ch12.Tg (12Tg) and wild-type Ch12 (12) were distinguished in separate experiments by Y116 hybridization. Overlapping *Xist* and Ch12 signals are yellow-white. Scale bar, 5 μ m.

in transgenics (48 of 69). However, a significant portion of transgenic cells expressing *Yy1* did so from Ch12.Tg (21 of 69; Fig. 3b, c). This implies that there was escape from *Yy1* inactivation in some cells.

The results of quantitative RT-PCR and RNA FISH indicate downregulation of genes *in cis* to the transgene suggestive of transcriptional inactivation. This is consistent with our previous finding that *lacZ*, a marker carried on the Y116 vector, was silenced in 116.6 fibroblasts⁵. If silencing mediated by ectopic *Xic*s resembled X inactivation, the transgenic autosome may acquire biochemical changes found on the *X_i*. Delayed replication timing is strongly correlated with gene silencing in yeast, *Drosophila* and mammals, in which the two events are controlled by the same elements¹⁴. The *X_i* replicates later in S phase than euchromatin and replicates asynchronously with its active homologue¹⁵. We tested whether the transgene altered Ch12.Tg replication by incorporation of bromodeoxyuridine (BrdU) during DNA synthesis. To identify the late S-phase window, we pulse-labelled non-synchronized control cultures with BrdU for 30 min at various time intervals before the cells were

collected. Cells pulsed at -7 h labelled autosomes and the active *X_i*, whereas cells pulsed at -4 to -6 h show labelling of the *X_i* and *Y*, thus establishing that late S occurs at -4 to -6 h.

In 32 transgenic metaphases examined from three independent lines during this S-phase window, two distinct chromosomes were labelled (Fig. 4). Ch12 and Y116 hybridization indicated that Ch12.Tg was uniquely labelled in 25 of 32 spreads. In three metaphases, the *Y* (identified by DAPI appearance) was solely labelled. In the remaining four spreads, both Ch12.Tg and the *Y* incorporated BrdU. Because the *Y* also replicates late in S¹⁶, co-labelling of the *Y* and Ch12.Tg independently confirmed delayed replication for Ch12.Tg (however, because a 30-min pulse identified only a narrow window in S phase (5 h), *Y* and Ch12.Tg did not always label together). No other chromosome, including wild-type Ch12, demonstrated delayed BrdU incorporation. These results indicate that, like the *X_i*, Ch12.Tg replicates late and asynchronously with its homologue.

A direct relationship between histone acetylation and transcription has been demonstrated, as acetylation of histone H4 correlates strongly with gene activity^{17,18}. In mammals, the female *X_i* is hypoacetylated at all four amino-terminal lysines¹⁹. To determine whether H4-deacetylation occurs on Ch12.Tg, metaphase chromosomes from 116.6 ES cells and fibroblasts were immunostained with antibodies R17 (which binds all four acetylated isoforms) and R41/5 (which binds lys-5-acetylated isoform)¹⁹. In undifferentiated transgenic ES cells, no chromosome was detectably hypoacetylated (Fig. 5); in contrast, two chromosomes were hypoacetylated in 90% of tetraploid fibroblast nuclei (Fig. 5a, b, e). When five independently cloned lines were examined, Ch12 painting and Y116 probes revealed that the underacetylated chromosome was invariably Ch12.Tg, and that the wild-type homologue achieved acetylation

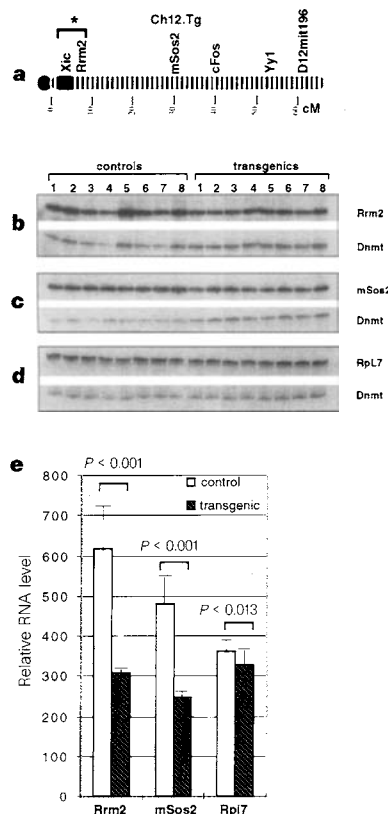


Figure 2 Gene expression from Ch12 is reduced twofold. **a**, Genetic map of Ch12 indicating approximate locations of housekeeping genes and microsatellite markers used in this study. Asterisk indicates that the relative positions of *Rrm2* and the transgene have not been established. **b-d**, Quantitative RT-PCR of *mSos2*, *Rrm2* and *Rpl7* (Ch2), coamplified with *Dnmt* (Ch9). Eight clones of fibroblast 116.6 and control isogenic fibroblast lines were used for analysis, none of which were aneuploid for Ch2, 9 and 12. PCR was performed in the exponential phase and all product levels were standardized to *Dnmt*. To ensure that only cDNA and not genomic DNA was amplified, RT-PCR was performed on +RT and -RT samples; -RT controls did not amplify (data not shown). **e**, Summary of relative gene expression. Averages and standard deviations were as follows, given as mean (s.d.): *Rrm2*, control 618 (110), Tg 306 (9). *mSos2*, control 481 (84), Tg 247 (17). *Rpl7*, control 361 (29), Tg 326 (46). The Mann-Whitney rank-sum test was used to determine statistical significance of the differences (*P*-values shown above each pair).

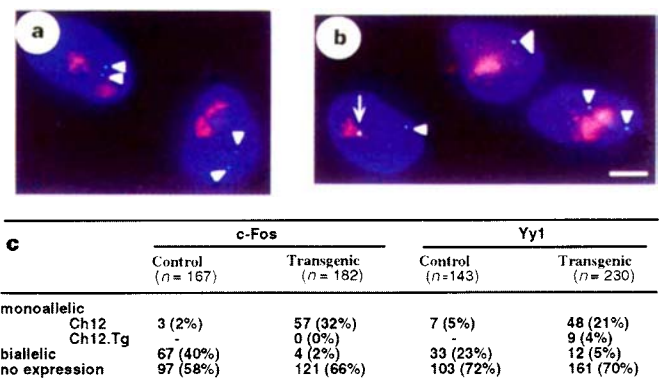


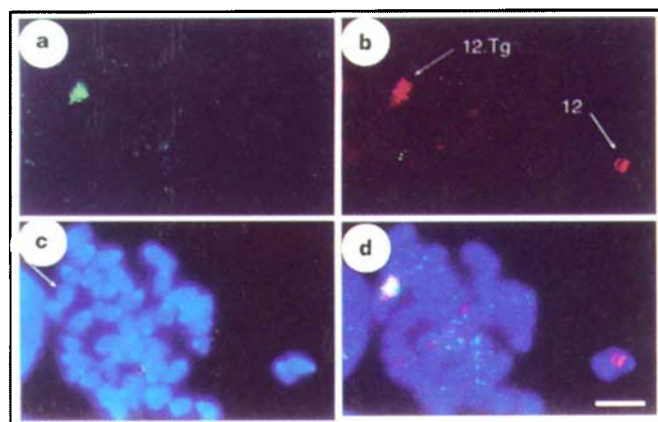
Figure 3 *c-Fos* and *Yy1* are monoallelically expressed in 116.6 fibroblasts. **a, b**, Serum-induced 116.6 fibroblasts hybridized to Texas-red-labelled *Xist* and FITC-labelled *c-Fos* (**a**) or *Yy1* (**b**). To visualize *c-Fos* and *Yy1* nascent transcript, a 4.4-kb murine *c-Fos* genomic probe (p302-356 pBS(-))¹⁵ and 1.8-kb murine *Yy1* cDNA probe (p8)²⁰ were used. Double photographic exposures were taken to determine the relative positions of nascent transcripts to *Xist* RNA. Ploidy was inferred from number of *Xist* signals; the reliability of the method was determined in separate experiments which established that diploids have one *Xist* signal and tetraploids have two. **a**, Arrowheads indicate *c-Fos* transcription from wild-type Ch12 (cells are tetraploid). **b**, Arrowheads indicate *Yy1* transcription from wild-type Ch12; the arrow indicates expression from Ch12.Tg (note both diploid and tetraploid nuclei in this field). Scale bar, 5 μ m. **c**, Distribution of monoallelic and biallelic expressors for *c-Fos* and *Yy1* in controls and transgenics; *n* is the sample size.

equal to that of other autosomes. In a minority (~10%) of some clonal cultures, the transgenic autosome was hypoacetylated on only the proximal half (Fig. 5c–e), which suggests that there must be some instability in the maintenance of chromatin structure. This is consistent with our finding that *Yyl* escapes inactivation in some cells (Fig. 3c). Although the Y was late replicating, it was not consistently underacetylated (2 out of 49; Fig. 5c, d). These data indicate that differentiation resulted in autosomal hypoacetylation similar to that of the X_i .

Our results therefore suggest that the *Xic* transgene affects chromatin structure and gene expression over a distance of 50 cM of the autosome. The ectopic loci enable Ch12 to exist either as an early-replicating, acetylated and presumably active chromosome, or as a late-replicating, hypoacetylated and probably inactive chromosome. These results imply that the *Xic* not only initiates X inactivation⁵ but also drives long-range heterochromatin formation. Furthermore, neither binding to *Xist* RNA nor the potential for chromosome-wide gene regulation is intrinsic to the X. Thus inactivation in our transgenic system does not absolutely require X-specific elements in propagation and/or stabilization of heterochromatin, and indicates that an *Xic in cis* is the primary requirement for the inactivation process. This contrasts with dosage compensation in *Drosophila*, which depends on such X-specific elements; autosome segments inserted into the male X are not dosage compensated, and X-linked genes inserted into autosomes remain partly compensated²⁰. However, this is consistent with mouse transgenic experiments, in which autosomal genes inserted into the X became subject to inactivation²¹ and, conversely, where X-linked genes inserted into autosomes were not dosage compensated²². Overt differences with human and mouse X-autosome translocations where spread of autosomal inactivation is variable and limited^{23,24}

could reflect *in vivo* selection against cells in which complete autosomal inactivation led to haplo-insufficiency.

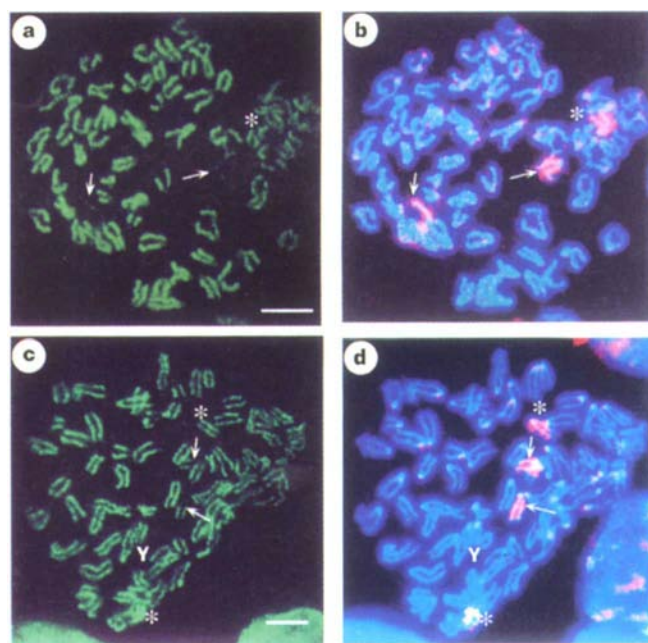
Our results demonstrate that long-range *cis* effects occur on autosomal DNA, but further work is required to determine whether silencing includes all autosomal genes. Furthermore, as some genes on the X_i are only partly inactivated^{25,26}, it is not yet clear whether *Xic*-mediated inactivation results in complete transcriptional repression of affected genes. *Xic*-mediated effects on autosomes may be less stable than on the X chromosome. Indeed, our study suggests that there is instability of either propagation or maintenance of heterochromatin on Ch12, as demonstrated by reacetylation of distal Ch12.Tg and sporadic escape of *Yyl* expression. Our unpublished results on other Y116 transgenics containing ~6 copies integrated into Ch10 (116.7)⁵ or 2–3 copies subtelomerically on Ch11 (116.13)⁵ suggest there are further complexities in the silencing mechanism. As differentiating ES cells, *Xist* RNA was expressed abundantly and showed *cis*-association with Ch10 and Ch11. However, expression and associated *cis*-effects were selected against in fibroblasts isolated from chimaeric mice (so this study could only be performed on 116.6). Varying sites of integration, *Xic*



e

BrdU-labelled chromosomes per metaphase	n
Ch12.Tg only	25
Y only	3
Ch12.Tg + Y	4
Other chromosomes	0

Figure 4 Ch12.Tg replicates late in S phase and asynchronously with its homologue. The 116.6 fibroblasts were pulse-labelled with BrdU for 30 min in the late S-phase window. **a**, Indirect immunofluorescence with anti-BrdU antibodies (Sigma) to detect BrdU-labelled chromosomes. **b**, FISH analysis with Ch12-paint detected by avidin-Texas red. Wild-type Ch12 (12) and Ch12.Tg (12.Tg) were distinguished by hybridization to Y116 (not shown). **c**, DAPI-counterstained chromosomes. Arrow, Ch12.Tg. **d**, Merging of **a–c**. **e**, The frequency with which Ch12.Tg and the Y were BrdU-labelled during late S phase. Scale bar, 5 μm.



e

Ch12.Tg acetylation pattern	ES cells	Fibroblasts
12	26	0
12	0	45
12	0	4

Figure 5 Histone H4 is hypoacetylated on Ch12.Tg. Metaphase chromosomes from 116.6 fibroblasts were immunolabelled with R17 (recognizing all acetylated H4 isoforms) or R41/5 (specific for acetylated lys-5 isoform)¹⁹ and indirectly detected by FITC. As results were similar with both antibodies, data are shown for only R17. **a**, Tetraploid metaphase chromosomes showing two hypoacetylated chromosomes (arrows), revealed in separate experiments to be Ch12.Tg by Y116 FISH (not shown). **b**, Same metaphase counterstained with DAPI and hybridized to Ch12 paint. Arrows, underacetylated Ch12.Tg; asterisks, fully acetylated wild-type Ch12. The second wild-type Ch12 is outside the field shown. **c**, In a minority of cells from some clones, Ch12.Tg is hypoacetylated only on the proximal half (arrows). Note the Y is also underacetylated. **d**, DAPI staining and Ch12 painting of the metaphase in **c**. **e**, Acetylation patterns and frequencies in 116.6 ES cells and fibroblasts. Stippled regions, acetylated. Scale bar, 5 μm.

copy number, and choice of cell types could all explain the observed differences. Future work is required to address whether *cis* effects result from *Xist* action alone or require additional function from the *Xic*.

Methods

Quantitative RT-PCR. Total cellular RNAs from mitotically active transgenic and control fibroblasts were prepared using RNAzol B (TelTest) as recommended. After DNaseI digestion, first-strand cDNA was synthesized from 2 µg of RNA⁵. For RT-PCR, 0.25 µg of cDNA was amplified with 5 U Taq polymerase (Promega), 1.5 mM MgCl₂, 200 µM dNTPs, and 250 nM each of *Dnmt* primers, *Sal5'* and *Sal3'B'* and primers for *RpL7*, *Rrm2* and *mSos2* as follows: *RpL7A*, 5'-GAAGCTCATCTATGAGAAGGC-3'; *RpL7B*, 5'-AAGACGAAGGAGCTGCAGAAC-3'; *Rrm2A*, 5'-AAGCGACTACCCCTGGCTGAC-3'; *Rrm2C*, 5'-GACTATGCCATCACTCGCTGC-3'; *Sos2B*, 5'-CTGGCCATGATTGGGTTTACA-3'; *Sos2C*, 5'-TATGTCCTCCACGCTCATG-3'. DNAs were denatured at 95 °C, 45 s; annealed at 55 °C, 45 s; and extended at 72 °C, 90 s. For quantification, primers *Sal3'B*, *Rrm2C*, *Sos2C*, and *RpL7B* were phosphorylated with polynucleotide kinase and [γ -³²P]ATP. PCR products were analysed in 6% polyacrylamide/8 M urea gels and quantified by phosphorimaging (Fujix BAS2000 BioImaging Analyzer and MacBas v2.2). Standard PCR curves for all genes were generated, indicating exponential amplification between 10 and 23 cycles; 20 cycles was chosen for all reactions. For the standard curve, a master mix of reagents was apportioned equally among 16 reaction tubes; PCR of 0.25 µg of control female cDNA was sampled every two cycles from 10–40 cycles.

FISH. Interphase nuclei and chromosome spreads for FISH were prepared by cytogenetic²⁷ or *in situ*²⁸ techniques. Probes were prepared by random priming with digoxigenin-11-dUTP or biotin-16-dUTP (Boehringer) and detected with anti-digoxigenin:rhodamine (Sigma) or avidin:FITC (Vector). Mouse chromosome-specific painting was performed as recommended (Applied Genetics Laboratory). *Xist* RNA was detected by λ 13.7 probe encompassing exon 1 and the promoter, and controls for ensuring detection of RNA, not DNA, were performed⁵.

DNA replication timing. Cultures were pulse-labelled with BrdU for 30 min, treated with 10 µg ml⁻¹ colchicine for 2 h before collection, 3–8 h after labelling. Detection of BrdU was performed²⁹ on metaphase chromosomes prepared by standard technique²⁷. Spreads containing late-replicating chromosomes were photographed with the Zeiss Axioskop/MC80 camera on 1600 Ektachrome film. Their stage coordinates were noted for overlaying with FISH results. To identify late-replicating chromosomes, slides were washed 3 times in 4 × SSC/0.1% Tween 20 for 5 min, at 45 °C, denatured in 70 mM NaOH + 70% ethanol for 3 min dehydrated in 80% and 100% ethanol for 2 min each, and subjected to FISH using Y116 and Ch12 painting probes. FISH results were photographed and overlaid with BrdU images in Photoshop 3.0.

Histone H4 acetylation. Chromosomes for immunostaining were prepared³⁰, photographed and marked by stage coordinates. To identify hypoacetylated chromosomes, slides were washed 3 times in 4 × SSC/0.1% Tween 20, then denatured in 2 × SSC/70% formamide at 72 °C for 5 min, and standard FISH results were photographed and overlaid with hypoacetylated chromosomes in Photoshop 3.0.

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GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors

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AMPA glutamate receptors mediate the majority of rapid excitatory synaptic transmission in the central nervous system^{1,2} and play a role in the synaptic plasticity underlying learning and memory^{3,4}. AMPA receptors are heteromeric complexes of four homologous subunits (GluR1–4) that differentially combine to form a variety of AMPA receptor subtypes^{1,2}. These subunits are thought to have a large extracellular amino-terminal domain, three transmembrane domains and an intracellular carboxy-terminal domain⁵. AMPA receptors are localized at excitatory synapses and are not found on adjacent inhibitory synapses enriched in GABA_A receptors⁶. The targeting of neurotransmitter receptors, such as AMPA receptors, and ion channels to synapses is essential for efficient transmission^{7,8}. A protein motif called a PDZ domain is important in the targeting of a variety of membrane proteins to cell–cell junctions including synapses^{8–10}. Here

we identify a synaptic PDZ domain-containing protein GRIP (glutamate receptor interacting protein) that specifically interacts with the C termini of AMPA receptors. GRIP is a new member of the PDZ domain-containing protein family which has seven PDZ domains and no catalytic domain. GRIP appears to serve as an adapter protein that links AMPA receptors to other proteins and may be critical for the clustering of AMPA receptors at excitatory synapses in the brain.

The C termini of Shaker-type K⁺ channels and the NMDA receptor are believed to be involved in their subcellular targeting through their interaction with the synaptic cytoskeletal protein PSD-95/SAP90^{9,10}. The structure of PSD-95/SAP90^{11,12} includes three repeats in the N-terminal region of a newly identified protein motif called a PDZ domain^{11,13}, named after three proteins containing the motif PSD-95, Dig-A and ZO-1^{8,11,13-15}. PDZ domains are motifs of about 90 amino acids¹¹ present in other members of the PSD/SAP90 family and a variety of other proteins including protein tyrosine phosphatases and nitric oxide synthase (NOS)^{8,11-17}. PDZ domains mediate protein-protein interactions by interacting with the C termini of proteins and are thought to be important in the subcellular targeting of the interacting proteins^{8,15,16}. The common element, referred to as a t/SXV motif^{9,10}, present in the C termini of NMDA receptor subunits (IESDV*) and K⁺ channels (VETDV*) which interact with the PDZ domains of PSD-95/SAP90 consists of a serine or a threonine followed one amino acid later by a valine. Although the C termini of AMPA receptor subunits do not contain the t/SXV motif, the C termini of the GluR2 and GluR3 subunits (IESVKI*) (asterisk indicates stop codon) do have weak homology to the C termini of NMDA receptors and K⁺ channels, suggesting that they may directly interact with PDZ domain-containing proteins. To identify proteins that interact with the C terminus of the GluR2 subunit we used the C-terminal 50 amino acids of GluR2 as bait to screen a rat hippocampal complementary DNA library using the yeast two-hybrid technique^{18,19}. Eight overlapping clones of a novel protein encoding three PDZ domains (PDZ4-6) were isolated. The interaction between this protein and the GluR2 terminus in the yeast two-hybrid system was specific, because cotransformations of

the isolated cDNAs with the Gal4 DNA-binding domain alone or with the C terminus of GluR1 were not positive (data not shown). We have called this protein GRIP for glutamate receptor interacting protein. Isolation of the full-length GRIP cDNA demonstrated that it is nearly 6 kb long with a large open reading frame of 3,336 bp. The sequence encodes a 1,112 amino-acid protein with a predicted relative molecular mass (*M_r*) of 120,350 (Fig. 1). Intriguingly, the full-length GRIP cDNA encodes seven PDZ domains (Fig. 1a, b). Sequence alignment of GRIP with other proteins that include PDZ domains indicated that each PDZ domain in GRIP contains GLGF-like repeats, a characteristic of PDZ domains, as well as other conserved amino acids (Fig. 1a). However, GRIP is a novel PDZ domain-containing protein which, in contrast to PSD-95/SAP90, NOS and the protein tyrosine phosphatases, does not contain any apparent catalytic domain. These results suggest that GRIP may crosslink AMPA receptors or serve as an adaptor or scaffolding protein to attach AMPA receptors to other proteins.

The yeast two-hybrid system was used to examine the domains of GRIP and GluR2 that are responsible for the GRIP-GluR2 interaction. Various constructs of the original GRIP isolate (PDZ4-6) were made that contained different combinations of the PDZ domains (see Fig. 1c). The shortest construct that was positive when paired with the GluR2 C terminus contained PDZ4, PDZ5 and about 30 amino acids on the N-terminal side of PDZ4, demonstrating that the other PDZ domains are not necessary for interaction with GluR2. It is not clear at this time why individual PDZ domains do not work in isolation; however, recent studies on the PDZ domain of NOS have indicated that sequences outside the PDZ domain are required for interaction with substrate peptides²⁰. Interestingly, PDZ123 and PDZ7 do not appear to interact with the C terminus of GluR2 in yeast although they do interact with other protein ligands (data not shown), indicating that the various PDZ domains in GRIP may have different specificities for peptide substrates. Deletion of the last seven amino acids of GluR2 completely disrupted the association of GRIP with GluR2 in this system demonstrating that the C-terminal seven amino acids of GluR2 are required for interaction (data not shown).

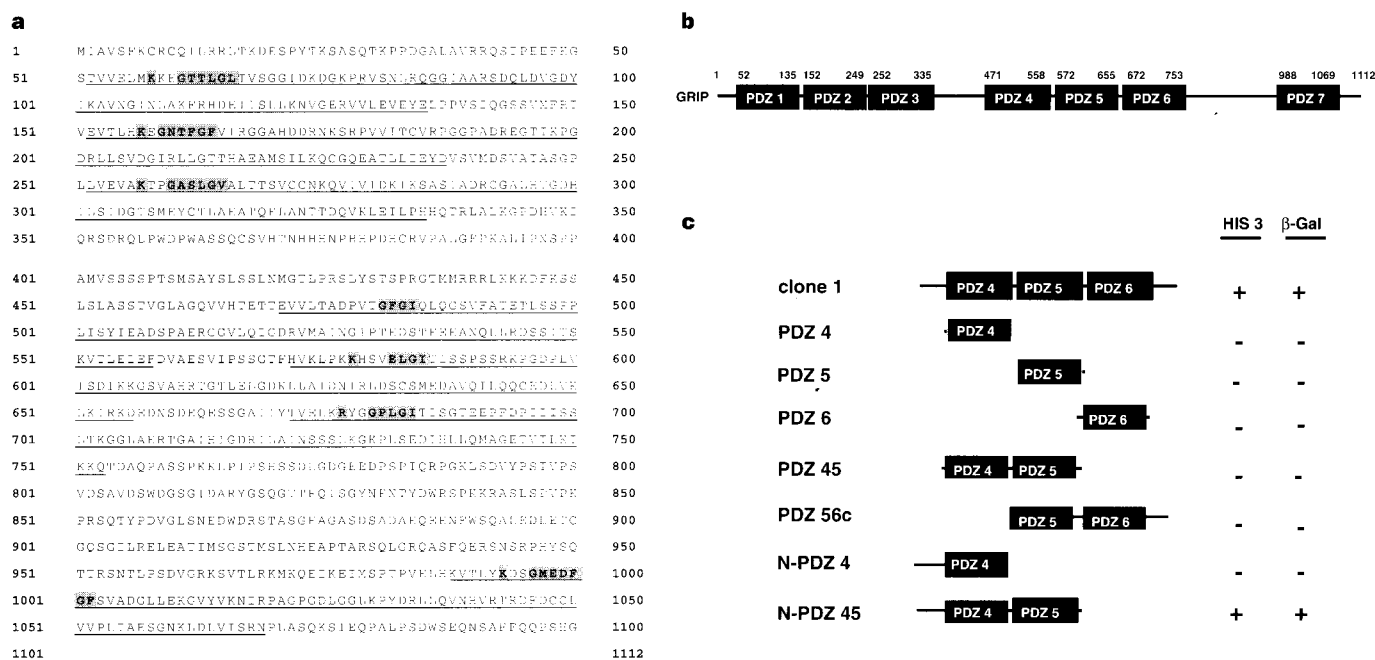


Figure 1 Structure of GRIP. **a**, Amino-acid sequence of GRIP. GRIP is a 1,112 amino-acid protein that contains seven PDZ domains and no obvious catalytic domain. The PDZ domains are indicated by underlining, the GLGF-like repeats and the conserved arginine/lysine residues are indicated by shading. **b**, Schematic of the

domain structure of GRIP. **c**, Structural determinants of GRIP required for interaction. The yeast two-hybrid system was used to test the domains of GRIP required for interaction with GluR2. Positive selection on His-plates and β-Gal activity of each construct is indicated.

To examine the interaction between the GluR2 subunit and GRIP in systems other than yeast, we subcloned the cDNA encoding PDZ domains 4–6 of GRIP into a mammalian expression vector with a myc-tag in the N terminus (myc-GRIP456) and cotransfected the myc-GRIP456 with the full-length GluR2 subunit into HEK-293 cells. These transfected cells were then solubilized with 1% Triton X-100 and the soluble cell lysate was immunoprecipitated with an anti-myc antibody (anti-myc). The resulting immunoprecipitates were then immunoblotted with an affinity-purified anti-GluR2/3 antibody that recognizes both GluR2 and GluR3. As shown in Fig. 2a, GluR2 could be immunoprecipitated by the anti-myc antibody only when it was cotransfected with GRIP, demonstrating that GluR2 associates with GRIP in this system. Because the GluR3 subunit has a similar C-terminal amino-acid sequence to GluR2, we examined whether GluR3 would also associate with GRIP in this system. Cotransfection of GluR3 with myc-GRIP456 also resulted in the coimmunoprecipitation of GluR3 with GRIP (see Fig. 2a). In contrast, the GluR1 subunit, which does not have a similar C-terminal amino-acid sequence, does not associate with GRIP under these conditions (Fig. 2b).

We also examined the interaction of GRIP with C-terminal mutants of the GluR2 subunit in transfected HEK-293 cells. To see if the C terminus was required for interaction, the last seven amino acids of GluR2 were deleted. In addition, a mutant in which the serine residue within the last seven amino acids (Ser 880) was mutated to an alanine was also tested. As described above for the

yeast two-hybrid system, deletion of the last seven amino acids eliminated the interaction of GRIP with the GluR2 subunit in the HEK-293 cells (Fig. 2c). Interestingly, mutation of serine 880 also completely eliminated the GRIP–GluR2 interaction (Fig. 2c), suggesting that similarly to the interaction between substrate peptides and the PDZ domains of PSD-95/SAP90^{8–10,21}, a serine (or threonine) residue is important for the interaction of GRIP PDZ domains with GluR2. The importance of this serine residue may indicate a potential role for protein phosphorylation in the regulation of GRIP–GluR2 interaction. In addition, the C termini of the GluR2 and GluR4 subunits have been shown to be alternatively spliced to give both a long form, like GluR1, and a short form like GluR3^{22,23}. This suggests that alternative splicing of the C terminus of AMPA receptor subunits may play a regulatory role in the clustering of AMPA receptors similar to that seen with the NMDA receptor^{9,24}.

To analyse the expression of the GRIP protein, an anti-fusion protein (amino acids 798–904) antibody and an anti-peptide (amino acids 646–664) antibody were generated against GRIP. The affinity-purified anti-fusion protein antibody recognized a single 130K protein in cells transfected with the full-length GRIP cDNA (see Fig. 3a). Interestingly, this antibody specifically recognized a 130K protein as well as an additional protein with an apparent M_r of 90K in rat brain (see Fig. 3a). These results suggest that the anti-GRIP fusion protein antibody crossreacts with another protein, possibly a GRIP-related protein, or alternatively, it recognizes a

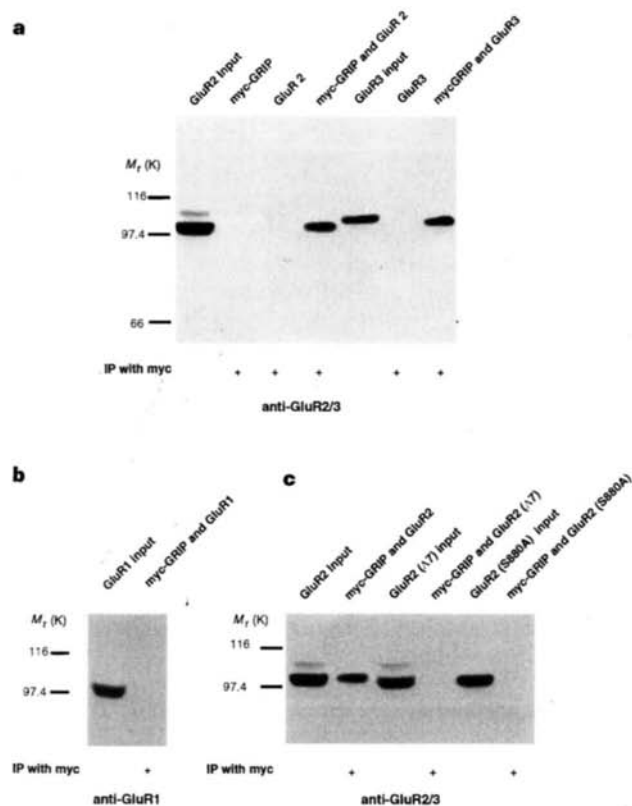


Figure 2 Coimmunoprecipitation of GRIP with GluR2 and GluR3. **a**, The full-length GluR2 or GluR3 subunits were expressed with or without myc-GRIP456 (as indicated) in HEK 293T cells. The cells were then solubilized with Triton X-100 and the myc-GRIP456 immunoprecipitated with an anti-myc antibody. The immunoprecipitates were then analysed by immunoblot techniques using an antibody that recognizes both GluR2 and GluR3. **b**, The GluR1 subunit and **c**, various mutants of GluR2 were also analysed as indicated.

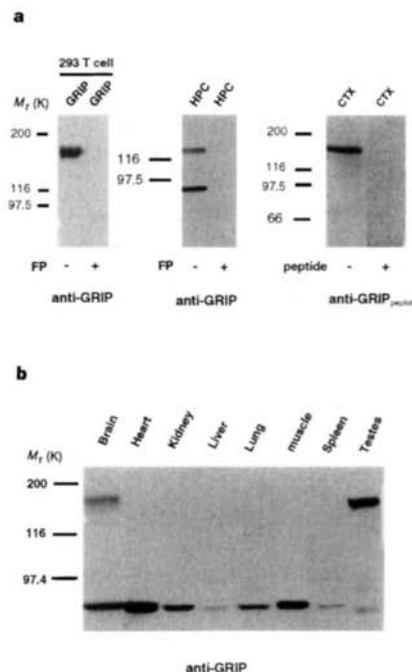


Figure 3 Distribution of GRIP protein in rat tissues. Antibodies were generated against a GRIP fusion protein and peptide, and used to identify the GRIP protein in rat tissues. **a**, Both the anti-fusion protein and the anti-peptide antibody recognized a 130K protein in HEK 293T cells transfected with the full-length GRIP cDNA, and in rat hippocampus. The anti-fusion protein also specifically recognized a 90K protein in hippocampus. Immunorecognition of these proteins by the antibodies was blocked by the immunogen (+FP, +Peptide). **b**, Immunoblot of various tissues with the anti-fusion protein antibody demonstrates that the 130K protein is only expressed in brain and testes whereas the 90K protein is expressed in most tissues examined.

proteolytic breakdown product of GRIP. The anti-peptide antibody also recognized the 130K protein in cells transfected with full-length GRIP cDNA (data not shown) and in rat brain (Fig. 3a) but did not recognize the 90K protein in brain (Fig. 3a). Attempts to coimmunoprecipitate GRIP with AMPA receptors from detergent extracts of rat brain have so far been unsuccessful because of the lack of solubility of GRIP in non-denaturing detergents (data not shown).

Using the anti-GRIP fusion protein antibody, the distribution of GRIP in neuronal and non-neuronal tissues was examined. The 130K protein seems to be brain-specific and was not detected in most tissues examined except testes (Fig. 3c). In contrast, the 90K protein was expressed in most tissues examined. GRIP was expressed in various brain regions including the olfactory bulb, cerebral cortex, hippocampus, cerebellum and spinal cord (data not shown). Northern blot analysis detected a ~6 kb GRIP messenger RNA which was only observed in brain and testes (data not shown).

To examine the subcellular distribution of GRIP in neurons, primary cultures of rat hippocampal neurons were stained with the anti-GRIP fusion protein antibody and visualized with a FITC-coupled secondary antibody. GRIP immunoreactivity was observed throughout the dendrites of hippocampal neurons and was clustered in many but not all neurons (Fig. 4a). AMPA receptors in hippocampal cultures cluster at excitatory synapses and consist of heteromeric complexes of the GluR1, GluR2 and GluR3 subunits⁶.

To see if AMPA receptors colocalized with GRIP clusters, we double-labelled the neurons with an antibody to the GluR1 subunits. As shown in Fig. 4, many clusters of GRIP colocalized with AMPA receptors at excitatory synapses. Although clustering of GRIP was seen in some neurons, not all AMPA receptor clusters contained high levels of GRIP staining. This may be due to a limited accessibility of the antibody to GRIP in the postsynaptic density, or alternatively, GRIP may be selectively localized at certain excitatory synapses. Antibodies against SAP102, a member of the PSD-95/SAP90 family, also selectively label excitatory synapses²⁵. By analogy with SAP102, it is likely that there will be additional members of the GRIP protein family that also may be involved in excitatory synaptic function. These results demonstrate that GRIP is present at excitatory synapses and suggest that GRIP may play a role in the synaptic targeting of AMPA receptors.

To determine whether GRIP may be involved in the clustering of AMPA receptors at excitatory synapses, we transfected primary neuronal cultures with constructs containing only the C-terminal region of the GluR2 subunit in an attempt to disrupt the interaction of endogenous GluR2/3 subunits with GRIP. Neurons were transfected with myc-tagged cDNA constructs encoding the last 50–226 amino acids of the C termini of the GluR2 (GluR2C) or the GluR1 (GluR1C) subunits of the AMPA receptor, or the NR1 subunit (NR1C) of the NMDA receptor. The cultures were then fixed and

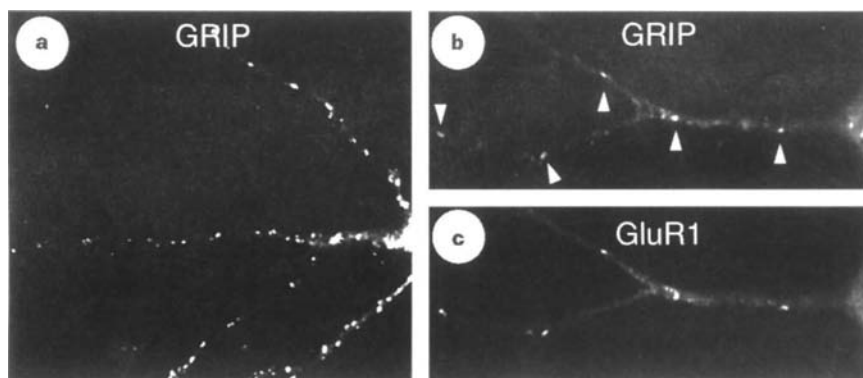


Figure 4 Colocalization of GRIP with AMPA receptors at excitatory synapses. Primary cultures of hippocampal neurons were double-labelled with anti-GRIP and anti-GluR1 antibodies. **a**, GRIP is present throughout neuronal dendrites and

is clustered on dendritic processes. A high-power magnification of a neuronal dendrite is shown in **b** and **c** with characteristic synaptic AMPA receptor clusters that colocalize with GRIP.

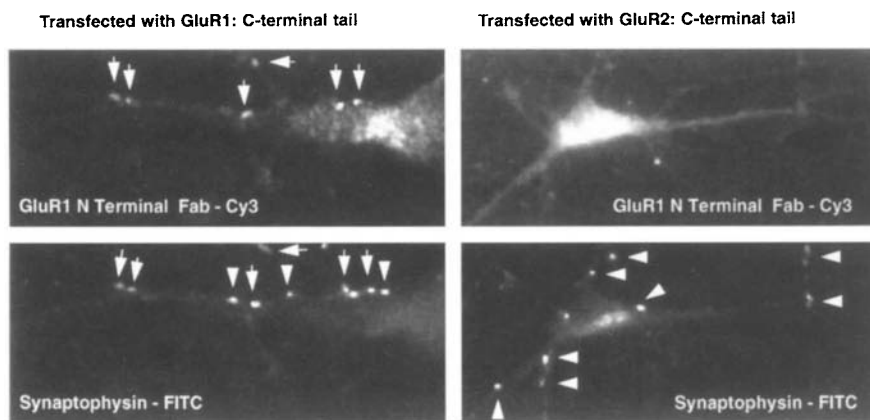


Figure 5 Disruption of synaptic AMPA receptor clustering by overexpression of the C terminus of GluR2 in neurons. In an attempt to disrupt the interaction of GluR2 with GRIP we transfected a myc-tagged construct of the last 50 amino acids of GluR2 (or as a control the C terminus of GluR1) into primary cultures of spinal cord neurons. The clustering of AMPA receptors was then analysed by immunocytochemical techniques. The transfected neurons were double-labelled with: (1) an antibody to synaptophysin to detect synapses and (2) a Cy3-labelled

FAB fragment of an antibody generated against an extracellular epitope of the GluR1 subunit to analyse AMPA receptor clustering. Synapses associated with AMPA receptor clusters are indicated with arrows and synapses without AMPA receptor clusters are indicated with arrowheads. Clustering of AMPA receptors was dramatically disrupted by the C terminus of GluR2 but not by the C terminus of GluR1.

triple labelled with (1) an anti-myc antibody to identify transfected neurons; (2) anti-GluR1 antibodies to identify AMPA receptor clusters within the transfected neurons (AMPA receptors in these cultures are heteromeric complexes of GluR1, GluR2 and/or GluR3, data not shown); and (3) the synapse-specific protein synaptophysin to identify synapses. Neurons transfected with GluR1C or NR1C have multiple synaptic AMPA receptor clusters which colocalize with the synaptic protein synaptophysin, similar to that seen in non-transfected neurons (Fig. 5: GluR1C, 3.6 ± 0.53 clusters per neuron ($n = 19$); NR1C, 3.5 ± 0.38 clusters per neuron ($n = 38$)). In contrast, when GluR2C was transfected into neurons, the number of synaptic AMPA receptor clusters dramatically decreased (Fig. 5: GluR2C, 1.2 ± 0.29 clusters per neuron ($n = 34$)). Expression of GluR2C, however, had no effect on the number of synaptic contacts detected with the anti-synaptophysin antibody or the presence of diffuse surface AMPA receptor staining (Fig. 5). To determine whether this disruption of AMPA receptor clustering by the GluR2 C terminus was dependent on the C-terminal seven amino acids that are required for interaction with GRIP, we deleted this region of the GluR2 subunit. Transfection of the C-terminal GluR2 construct, which lacked the last seven amino acids (GluR2C Δ 7), did not disrupt clustering of the AMPA receptor (GluR2C Δ 7, 2.9 ± 0.48 clusters per neuron ($n = 10$)). These results strongly suggest that expression of the C-terminal tail of GluR2 disrupts the interaction of AMPA receptors with a synaptic protein, most probably GRIP, which is important in clustering of AMPA receptors.

Clustering of postsynaptic neurotransmitter receptors is important for the efficiency of synaptic transmission^{7,8}. Recent studies of cellular models of synaptic plasticity such as long-term potentiation have suggested that the regulation of the synaptic targeting of AMPA receptors may be important for the modulation of synaptic function^{26–28}. Although GRIP appears to play a role in the synaptic targeting of AMPA receptors, the exact role of GRIP in AMPA receptor clustering is unclear. Studies on the NMDA receptor and K⁺ channels have suggested that the three PDZ repeats in the PSD-95/SAP90 family members could provide a mechanism for neurotransmitter receptor clustering by crosslinking receptors or anchoring them to the cytoskeleton^{8–10}. PSD-95/SAP90 family members have also been implicated in colocalizing signal transduction machinery with NMDA receptors near the postsynaptic membrane¹⁷. Neuronal nitric oxide synthase (nNOS) possesses a PDZ domain and can interact with PSD-95/SAP90 and PSD-93 through a PDZ-PDZ domain interaction¹⁷. As nNOS is activated by Ca²⁺ influx through NMDA receptors, their coupling via PSD-95/SAP90 could provide a possible mechanism for localized intracellular signalling upon activation of NMDA receptors. GRIP contains seven PDZ domains which may play a similar role to the PDZ domains in the PSD-95/SAP90 family by crosslinking AMPA receptors or linking them to the cytoskeleton or to signal-transducing enzymes. The multivalent nature of GRIP with its seven PDZ domains allows for a large diversity of potential interactions. Identification of these interacting partners will help to clarify further the mechanisms underlying the regulation of excitatory synapse formation and function.

The GenBank accession number for the GRIP protein and DNA sequence is U88572. □

Methods

Yeast two-hybrid system screening. Yeast two-hybrid screening¹⁸ was performed using a random-primed cDNA library from rat hippocampus subcloned into the *SalI/NotI* sites of the pPC86 vector¹⁹, which contains the GAL4 activation domain, and the final 50 amino acids of GluR2 subcloned in-frame into the *SalI/BglII* sites of the pPC97 vector, which contains the GAL4 DNA-binding domain. The plasmids were transformed into Y190 yeast cells²⁹ and positive clones were selected on triple-minus plates (Leu[−], Trp[−], His[−]) and assayed for β -galactosidase activity. Positive clones were cotransformed with either the bait vector or the original pPC97 vector (backbone) into yeast to

confirm the interaction. All the constructs that were used in other mating tests were from PCR products subcloned in-frame into pPC97 or pPC86 vectors and were confirmed by sequencing. Mutations in GluR2 constructs were performed using double-stranded site-directed mutagenesis (Chameleon system, Strategene) according to the manufacturer's instructions. Mutations were confirmed by sequencing.

HEK 293 cell transfection. cDNAs subcloned into the pBCKMV vector or in the pRK5 vector (20 μ g per 10 cm culture dish) with or without an N-terminal 16 amino acid myc-tag were transfected into HEK 293T cells using calcium phosphate coprecipitation as described²⁴. After transfection (36–48 h), HEK 293T cells were solubilized in immunoprecipitation (IP) buffer (25 mM Tris-HCl-buffered saline (pH 7.4), 10 units per ml Trasylol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM EDTA and 5 mM EGTA) and 1% Triton X-100. For coimmunoprecipitation of GRIP and GluR2 the cDNA containing PDZ domains 4–6 (amino acids 435–969) of GRIP (myc-GRIP456) were subcloned into the *SalI/NotI* sites of a myc-tagged pRK5 vector. HEK 293T cells cotransfected with GluR2 (15 μ g) and myc-GRIP456 (5 μ g) were solubilized with 1% Triton X-100 in IP buffer and the supernatant used for immunoprecipitation with an anti-myc antibody. The immunoprecipitated proteins were eluted with SDS sample buffer, separated using SDS-PAGE, transferred to PVDF and subjected to immunoblot analysis with antibodies to the C termini of GluR2/3 and GluR1.

Generation of GRIP antibodies. GRIP anti-peptide and anti-fusion protein antibodies were generated in rabbits. A GST-fusion protein corresponding to amino acids 798–904 of GRIP was used to produce antiserum. This antiserum was then affinity purified on a fusion protein affinity column. In addition, a 20 amino acid peptide (KEDLVKLRKDEDSNDEQE) corresponding to amino acids 646–664 of GRIP was synthesized, crosslinked to carrier protein and injected into rabbits to generate antiserum. The resulting antiserum was then affinity purified on a peptide affinity column.

GRIP immunocytochemistry. Cultured hippocampal neurons were fixed and stained as described⁶. Affinity-purified anti-GRIP fusion protein antibody (0.2 μ g ml^{−1}) was incubated overnight in 3% NGS followed by FITC anti-rabbit antibody. Cells were then incubated in 5% normal rabbit serum for 30 min followed by Cy3-labelled anti-GluR1 antibody in 5% rabbit serum overnight. Cultures were then mounted in Permafluor (Immunon) with 20 mg ml^{−1} Dabco and viewed at 100 $\times$. Appropriate controls using fusion protein and peptide blocking of the anti-GRIP and anti-GluR1 antibodies showed no staining of the neurons.

Transfection of spinal cord cultures. The C-terminal 50–226 amino acids of the GluR2 (GluR2C), GluR1 (GluR1C), and the NR1 (NR1C) subunits, or a mutant of the GluR2 C terminus lacking the last 7 amino acids (GluR2C Δ 7) were subcloned into the *SalI/NotI* sites of the myc-tagged pRK5 vector. Spinal cord neurons were taken from the ventral lumbar spinal cord of E 19 rat embryos using papain digestion. The cells were grown at low density (40 neurons per mm²) in 75% MEM, 25% Neurobasal Media (Gibco) supplemented with N2 supplements⁶, 2% horse serum, and 15 μ g ml^{−1} chick leg extract. On day 2 in culture the neurons were pretreated for 30 min with DMEM adjusted to an Osm of 280 supplemented with 1 mM kynurenate and transfected with 8 μ g of the various cDNA constructs using a modification of a previously published method³⁰. The neurons were triple-labelled with: (1) anti-myc antibody to detect neurons transfected with the myc-tagged proteins; (2) anti-synaptophysin antibodies to label synapses; and (3) anti-GluR1 antibodies to identify endogenous AMPA receptor clusters. The anti-GluR1 antibody used in this experiment was a Fab fragment of an antibody raised against a synthetic peptide corresponding to amino acids 251–269 in the N-terminal region of GluR1. Live neurons were incubated for 1 h with 10 μ g ml^{−1} Cy3-labelled Fab fragment in growth media, rinsed with MEM three times and fixed in 4% paraformaldehyde. Neurons were then labelled with a mouse monoclonal synaptophysin antibody (Boehringer Mannheim) followed by FITC-coupled anti-mouse secondary antibody. Finally, the cells were labelled with the anti-myc antibody and AMCA coupled anti-mouse secondary antibody.

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Homer: a protein that selectively binds metabotropic glutamate receptors

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Spatial localization and clustering of membrane proteins is critical to neuronal development and synaptic plasticity. Recent studies have identified a family of proteins, the PDZ proteins, that contain modular PDZ domains and interact with synaptic

ionotropic glutamate receptors¹ and ion channels². PDZ proteins are thought to have a role in defining the cellular distribution of the proteins that interact with them. Here we report a novel dendritic protein, Homer, that contains a single, PDZ-like domain and binds specifically to the carboxy terminus of phosphoinositide-linked metabotropic glutamate receptors. Homer is highly divergent from known PDZ proteins and seems to represent a novel family. The Homer gene is also distinct from members of the PDZ family in that its expression is regulated as an immediate early gene and is dynamically responsive to physiological synaptic activity, particularly during cortical development. This dynamic transcriptional control suggests that Homer mediates a novel cellular mechanism that regulates metabotropic glutamate signalling.

Rapid macromolecular synthesis is required for the establishment of long-term neuronal plasticity^{3,4}. To identify molecules that are involved in this process, we and others have used differential cloning strategies to identify genes that are rapidly induced in neurons of the hippocampus and cortex by excitatory synaptic activity^{5,6–10}. Here we report a novel gene, henceforth termed *Homer*, which is regulated by synaptic activity. *Homer* mRNA is 6.5 kb long and encodes a protein of 186 amino acids (Fig. 1a). A long 3' untranslated region encodes multiple AUUUA repeats, which are implicated in the instability of immediate early gene mRNAs¹¹. The amino-acid sequence predicts a soluble protein containing a single GLGF sequence and a preceding arginine (Fig. 1a), as seen in the binding pocket of the typical PDZ protein PSD-95¹². The sequence is otherwise novel and there is less than 10% amino-acid sequence identity between Homer and reported members of the PDZ family. Expressed sequence tags (ESTs) from human (Z17805) and mouse (AA166092 and AA013888) were identified that are 84% and 72% identical to regions of the *Homer* open reading frame (ORF) (Fig. 1a). Translation of the ESTs indicates that the amino-acid sequences of the mouse and human ESTs are identical to each other in their limited region of overlap but that the mouse ESTs are divergent from Homer (Fig. 1a), suggesting that the ESTs are homologues of an additional family member.

Expression of *Homer* mRNA is brain specific and is strongly upregulated in the hippocampus by seizure-induced neuronal activation, with peak mRNA expression occurring within 1 hour (Fig. 1b). Homer protein is enriched in extracts of hippocampus and migrates as a doublet with an apparent relative molecular mass (M_r) of 28,000/29,000 (28/29K) (Fig. 1c) that is rapidly induced by seizure. As a means of identifying possible functional partners of Homer, we performed a two-hybrid screen using a rat brain cDNA library. One of the candidate interacting cDNAs encodes the C-terminal 195 amino acids of the type 5 metabotropic glutamate receptor (mGluR5). This region of mGluR5 is cytosolic and may be important in signalling mediated by phospholipase C^{13,14}. We confirmed the biochemical interaction using *in vitro* binding and coimmunoprecipitation assays. Bacterially expressed GST-Homer fusion protein binds native mGluR5 in detergent extracts of hippocampus (Fig. 2a). Moreover, mGluR5 coimmunoprecipitates with Homer from rat hippocampus (Fig. 2b).

The specificity of the interaction between Homer and metabotropic glutamate receptors was examined using *in vitro* binding assays. mGluR1 and mGluR5 couple to phospholipase C and regulate phosphoinositide hydrolysis, while mGluR2 and -4 negatively regulate adenylyl cyclase^{13,14}. Metabotropic glutamate receptors mGluR5 and mGluR1 α uniquely possess long cytoplasmic C-terminal tails that are 67% identical over the last 55 amino acids and terminate in similar sequences (Fig. 3a). Consistent with this sequence homology, transiently expressed full-length mGluR1 α and mGluR5 both bind Homer fusion protein (Fig. 3b). Given the precedent that the interaction between PSD95 and NMDAR2 requires the C-terminal four amino acids of the receptor¹, we examined the effect of deleting the C-terminal four amino acids of mGluR5. This deletion reduced binding to Homer by >80%

a				
Homer	NGEQIFITR AHUFQIDPNT KKNMPASKH	30		
Human EST	NGEQIFITR AHUFQIDPNT KKNMPASKH	30		
Mouse EST	-----			
Homer	AUTUSYFVDS TANUYRIISL DSKAIINST	60		
Human EST	GAHF-VFVDU TANSVRIISU D-----	50		
Mouse EST	-----VFVDU TANSVRIISU DGAUKIINST	25		
Homer	ITPNMTFKT SQKFGQWADS RANTUFGGLF	90		
Human EST	-----			
Mouse EST	ITPNMTFKT SQKFGQWADS RANTUFGGLF	55		
Homer	SSEHLKSKFA EKQEFKEAR ALAKEKSQEK	120		
Human EST	-----			
Mouse EST	SSELQLTKFA EKQFEUEAR ALARDKSQEK	85		
Homer	MELTSTPSQE SAGGDLQSP LTPESINGTDD	150		
Human EST	-----			
Mouse EST	TETSSNHSQE S-GCEPSS LQASSUNGTTD	114		
Homer	EATPDUTQNS EPRAEPQNA LPFSHRYTFN	150		
Human EST	-----			
Mouse EST	EKASHASPAD THLKSENDKL KIALTQSAAN	144		
Homer	SRIMIK---	186		
Human EST	-----			
Mouse EST	UKKEMELQ	153		

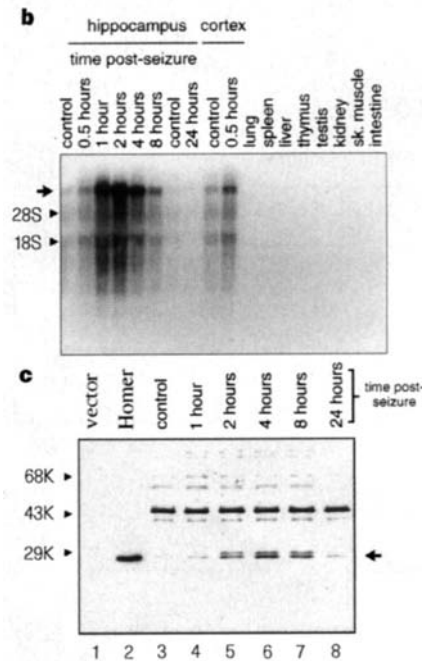


Figure 1 Cloning and expression of *Homer*. **a**, Predicted ORF and deduced amino-acid sequence of full-length *Homer* and ESTs from human and mouse (single-letter notation). Shaded sequences indicate areas of amino-acid identity. A bar above the sequence indicates the GLGF and preceding arginine, which suggest homology to PDZ-domain proteins¹². **b**, Northern blot analysis of total RNA (10µg) from rat brain and indicated organs. *Homer* mRNA (~6.5 kb; arrow) is rapidly and transiently induced in hippocampus following electroconvulsive seizure. Peak mRNA induction occurs by 1 h. *Homer* mRNA is markedly enriched

in brain relative to peripheral tissues. **c**, Immunoblot analysis of *Homer* protein. Full-length *Homer* expressed in HEK293 cells migrates as a 28K protein (lane 2) that is not present in cells transfected with vector alone (lane 1). Immunoblot of hippocampus from control and seizure stimulated rats (lanes 3–8) demonstrates that *Homer* migrates as a 28/29 doublet (arrow). *Homer* protein is maximally induced 4 h after a seizure. *Homer* was originally cloned by conventional differential screening of a phage cDNA library prepared from hippocampus of rats 4 h following electroconvulsive seizure as described previously⁷.

(Fig. 3b). Deletion of the C-terminal 10 amino acids completely abolished binding (not shown). Comparison of the C-terminal sequences of other metabotropic glutamate receptors indicates that mGluR2 and mGluR3 receptors share a similar C-terminal sequence—TSSL in the single-letter amino-acid notation (Fig. 3a), although they diverge from mGluR1α and mGluR5 outside this region. Neither mGluR2 nor mGluR4 bound *Homer* (Fig. 3b). We conclude that the C-terminal four amino acids are important, but not sufficient, for binding. The absence of a conserved C-terminal amino-acid sequence in mGluR1 β and γ or other group 3 metabotropic receptors indicates that they are unlikely to interact with *Homer*, although this remains to be examined directly.

We next examined the effect of deletion mutants of *Homer* on its binding to mGluR5. Full-length *Homer*-GST fusion protein bound a myc-tagged mGluR5 C-terminal 195 amino-acid fragment expressed from HEK293 cells (Fig. 3c). Similarly, a deletion construct lacking the C-terminal 55 amino acids also bound mGluR5. By contrast, deletion of the amino-terminal 108 amino acids of *Homer*, which includes the GLGF sequence, abolished binding to mGluR5. These observations indicate a role for the GLGF region in binding to the C-terminal sequence of mGluR5.

The anatomic and cellular pattern of *Homer* protein expression was examined by immunohistochemical analyses in adult rat brain. Consistent with its regulation as an immediate early gene, *Homer* immunostaining in cortex markedly increased 4 h following a seizure (Fig. 4a, b). The anatomic and temporal pattern of *Homer* immunostaining precisely paralleled its mRNA expression. Pyramidal neurons of cortical layers II/III and V demonstrated most intense immunostaining, which typically filled the soma and extended into apical dendrites in a punctate pattern along the perimeter of the dendrite (Fig. 4d). *Homer* immunoreactivity was not present in the nucleus. The punctate pattern of *Homer*

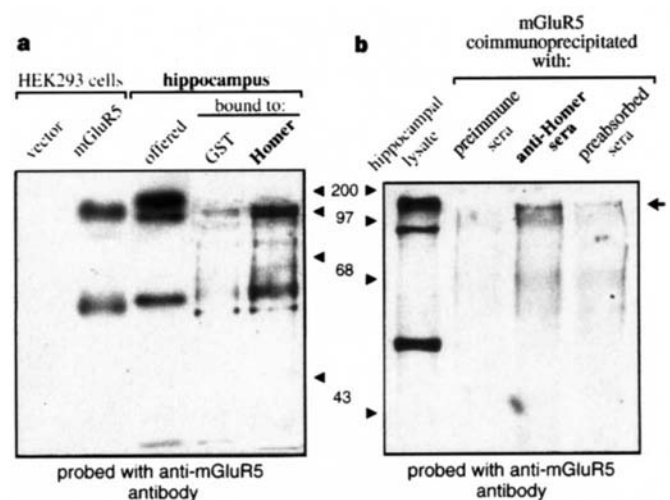


Figure 2 Interaction of *Homer* with mGluR5. **a**, *In vitro* binding. Immunoblot of mGluR5 expressed in HEK293 cells (lane 2) compared to cells transfected with vector alone (lane 1). mGluR5 migrates as a major band of ~140K with a secondary 50K presumptive cleavage fragment. In extracts of hippocampus (lane 3), the upper band appears as a doublet. Hippocampal extracts were passed over Affigel gel columns containing either GST (lane 4) or GST-Homer fusion protein (lane 5) and eluted with SDS loading buffer. mGluR5 binds to GST-Homer fusion protein, but not to GST. **b**, Coimmunoprecipitation of mGluR5 with Homer from hippocampus. Extracts of hippocampus were immunoprecipitated with either preimmune serum, anti-Homer serum or anti-Homer serum pretreated with GST-Homer. mGluR5 coimmunoprecipitates with Homer antiserum but not preimmune serum (arrow). Coimmunoprecipitation was blocked by preabsorption of antisera.

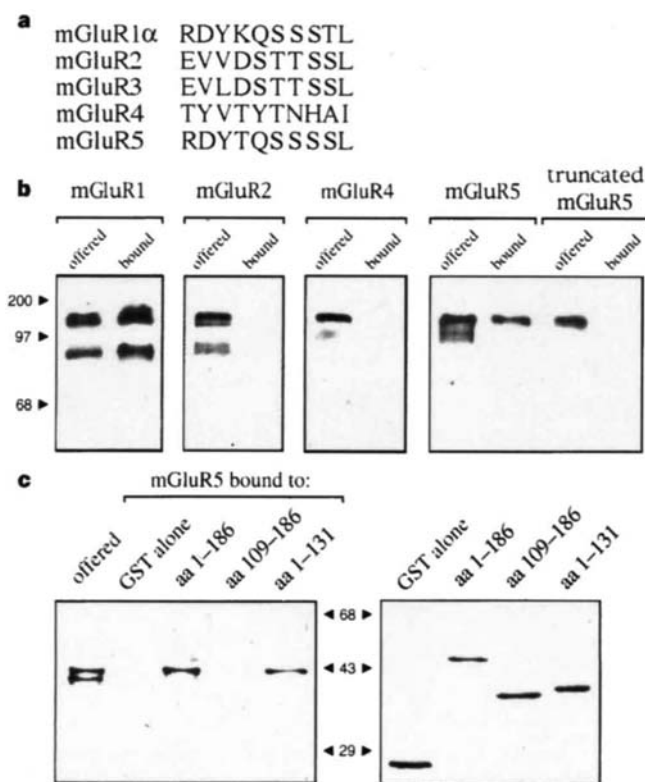


Figure 3 Homer selectively binds mGluR1 α and mGluR5. **a** The C-terminal 10 amino acids of metabotropic glutamate receptors 1 α and 2-5 are given. Note the similarity between mGluR1 α and mGluR5 and between mGluR2 and mGluR3. **b**, Immunoblot analysis of *in vitro* binding of metabotropic glutamate receptors to Homer. Receptors were expressed in HEK293 cells and extracts were mixed with bead-linked GST-Homer and eluted with SDS loading buffer. Offered extracts confirm expression for each receptor. Full-length mGluR1 α and mGluR5 bind Homer. Deletion of the C-terminal four amino acids of mGluR5 (truncated mGluR5) blocks binding. Homer does not bind either mGluR2 or mGluR4. **c**, *In vitro* binding assays were again used to examine deletion constructs of GST-Homer for binding to myc-tagged mGluR5 C terminus (195 amino acids) expressed in HEK293 cells. Lane markers indicate the portion of Homer expressed. The left panel was immunoblotted for myc and demonstrates mGluR5 binding to full-length Homer (1-186) and to fragment 1-131 but not 109-186. Right panel is Coomassie stain of Homer deletion constructs.

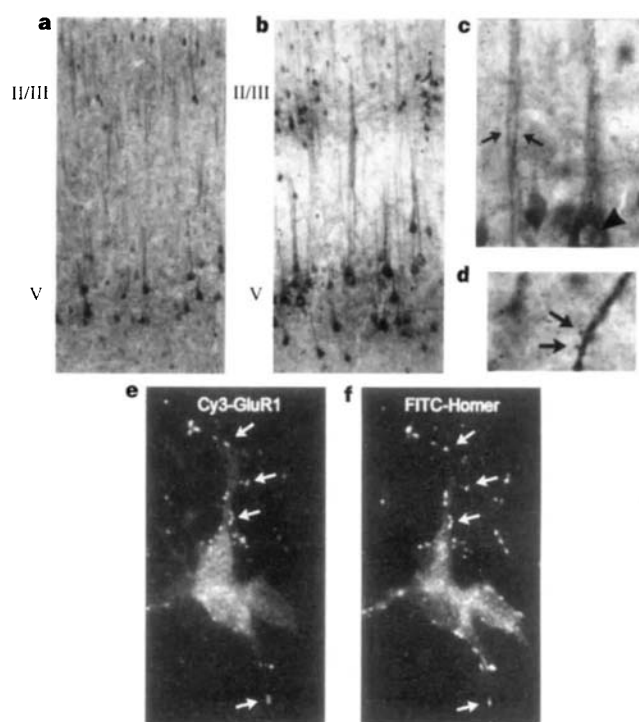


Figure 4 Homer protein is expressed in synaptic regions of neurons. Homer immunoreactivity in cortex of adult rats detected by peroxidase method (**a-d**); control (**a**) and 4 h after a seizure (**b**). Immunostaining is induced by seizure and is enriched in pyramidal neurons of layers II/III and V (magnification 100 $\times$). **c**, Immunoreactivity is present along dendritic shafts (arrows) and in cell bodies but not in the nucleus (arrowhead; 600 $\times$). Distal dendrites possess spine-like profiles (**d**, 1000 $\times$). Double immunofluorescent localization of AMPA-type glutamate receptor, GluR1, and Homer in neurons of primary hippocampal culture (**e, f**, 600 $\times$). Arrows indicate the punctate pattern of Homer staining that extensively colocalizes with GluR1.

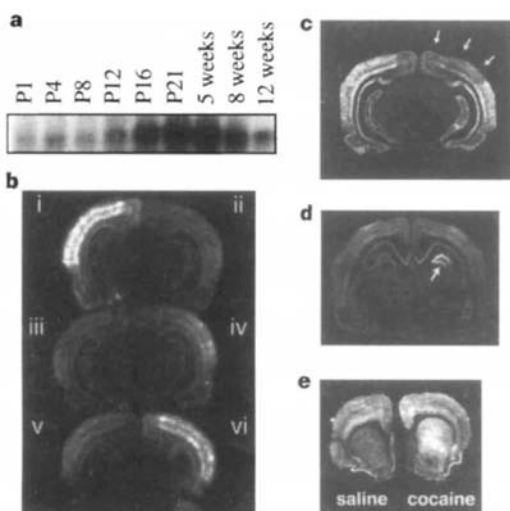


Figure 5 Regulation of *Homer* mRNA by development and physiological neuronal activity. **a**, Northern blot (10 μ g total RNA) demonstrating postnatal increase of *Homer* mRNA in rat forebrain. Peak expression occurs between 3-5 weeks. (**b-e**) *In situ* localization of *Homer* mRNA in coronal sections of rat brain. **b**, induction of *Homer* in cortex by visual experience. Dark-reared, 3-week-old rat pups were either killed in the dark (ii and iii) or exposed to ambient room light for 30 min prior to killing (i and vi). iv and v are age-matched control rats raised in normal diurnal conditions. Note rapid increase in *Homer* mRNA in cortex of dark-reared, light-exposed rats. **c**, Visual activity maintains *Homer* expression in cortex. A uniocular injection of tetrodotoxin (TTX) was made into the eye of 3-week-old rats 4 h before killing. *In situ* localization demonstrates reduction of *Homer* mRNA selectively in the visual cortex contralateral to TTX injection (arrows). **d**, Induction of *Homer* mRNA in hippocampal granule cells following a synaptic stimulus that produced LTP²⁸. **e**, *Homer* is rapidly induced in the striatum by administration of cocaine (10 μ g per kg i.p.) 2 h before killing¹⁹. Results were replicated in at least three independent experiments. Details of animal models are provided in the references.

immunostaining was confirmed in primary cultures of hippocampal neurons (Fig. 4f). Moreover, Homer extensively colocalized with immunostaining for the glutamate receptor GluR1, indicating that Homer is enriched at excitatory synapses. The anatomic pattern of Homer expression closely matches reports of mGluR5 immunoreactivity¹⁵. mGluR5 immunoreactivity is enriched in dendrites of cortical pyramidal neurons, as well as many other neuronal populations that express Homer, and is present at excitatory synapses. While technical limitations have prevented high-resolution colocalizations of Homer and mGluR5 in neurons, their extensive codistribution in pyramidal neuron dendrites and excitatory synapses, together with the striking specificity of their physical interaction, support the notion that they are physiological partners. Homer is also highly expressed in Purkinje cells of the cerebellum (not shown), which strongly express mGluR1 α ¹⁶, suggesting a physiological partnership in these neurons.

One of the most striking features of the *Homer* gene is its dramatic regulation by physiological neuronal activity. *Homer* is developmentally regulated, with peak expression in the rat forebrain from the third to fifth postnatal weeks (Fig. 5a). During this period of peak developmental expression, *Homer* mRNA is markedly induced in cerebral cortex of dark-reared rats within 30 min of the first visual experience (Fig. 4b). Moreover, monocular deprivation by blockade of retinal activity with tetrodotoxin causes a rapid reduction of *Homer* mRNA in the contralateral visual cortex (Fig. 4c). These observations indicate that its developmental expression in cortex is regulated by natural synaptic activity¹⁷. In the adult, *Homer* mRNA is rapidly induced in the hippocampus of awake, behaving, rats by NMDA-dependent synaptic stimuli that induce long-term potentiation (Fig. 4d). *Homer* is also rapidly induced in the striatum by cocaine (Fig. 4e), suggesting regulation by dopamine receptor mechanisms^{18,19}.

How might Homer contribute to neuronal function? The C terminus of mGluR1 α is known to be important in defining the efficacy of coupling to phospholipase C^{20–22}. By virtue of its interaction with the C terminus, Homer might modify signal transduction. Homer might, alternatively, play a role in spatial targeting of receptors. Both mGluR5 and mGluR1 α localize to discrete sites at postsynaptic excitatory synapses^{15,23,24}. The selective expression of Homer at excitatory synapses of relatively immature neuronal cultures supports a role in synapse formation. Mechanisms regulating its synaptic targeting remain to be determined. In our primary neuronal cultures, we do not detect clustered mGluR5 receptors, suggesting that Homer might localize by means other than its interaction with mGluR5.

Homer is exemplary of what appears to be a small set of immediate early genes in the brain^{5,6–10,25} that encode proteins distinct from conventionally recognized immediate early gene transcription factors in that they can directly modify cellular function. The unique regulation of *Homer*, its specificity for phosphoinositide-linked metabotropic glutamate receptors, its targeting to excitatory synapses and its novel PDZ-like domain suggest it will provide important insights into glutamatergic synaptic plasticity. □

Methods

Two-hybrid screen. Full-length Homer ORF with flanking *Sma*I sites was subcloned into the yeast expression vector pPC97²⁶. A random-primed cDNA library was prepared from seizure-stimulated adult rat hippocampus and cloned into the yeast expression vector pPC86²⁶. The library contains 6×10^6 independent cDNAs and a total of 1.5×10^6 were screened. Interacting proteins were identified by colony selection on plates lacking leucine, tryptophan, and histidine and confirmed using a β -galactosidase assay.

Antisera preparation and expression constructs. Rabbit polyclonal antisera for the mGluR receptors were generated against C-terminal peptides; mGluR1 as reported previously¹⁶, mGluR2/3 (Chemicon International Inc.), mGluR4 (Wyeth-Ayerst), and mGluR5 against a C-terminal 21-amino acid peptide.

Anti-Homer rabbit polyclonal antisera were generated using either the full-length Homer ORF as a GST fusion or a C-terminal 18-amino acid peptide. Both antisera detect a 28K protein when Homer is expressed in HEK293 cells and a seizure-inducible 28/29K doublet in hippocampus. The presented data use the peptide antiserum. A mammalian expression construct of full-length Homer was prepared by cloning the 5' *Eco*RI fragment (1.6 kb) into pRK5 (Genentech).

Immunohistochemistry. Six-week-old rats were anaesthetized and perfused with 4% paraformaldehyde. Whole brains were removed and placed in the fixative for 1 h and then into 30% Sucrose for 72 h. Sections (35 μ m) were cut using a sliding microtome, blocked and permeabilized for 1 h in 1% milk and 5% normal goat serum in PBS with 0.1% Triton. Sections were incubated in primary antibody for 24 h, washed, and immunoperoxidase staining was performed with a Vectastain Elite ABC Kit (Vector Laboratories). Elimination of the primary or preabsorption of Homer or mGluR5 antisera with the immunogenic peptides completely blocked staining.

Primary hippocampal cultures were prepared from 4-day postnatal rat pups as described²⁷. GluR1 staining was performed using Cy3-labelled Fab fragment of an antibody raised against synthetic peptide corresponding to amino acids 251–269 (ref. 28). Cells were fixed in 4% paraformaldehyde for 1 h, permeabilized with 0.1% Triton and incubated with affinity-purified anti-Homer antiserum overnight at 4 °C. Homer was detected by FITC-coupled goat anti-rabbit antibody (Vector Laboratories).

In vitro binding. Hippocampal lysate was prepared by sonicating hippocampi of 21-day-old rats (3×10 s) in PBS and 1% Triton with protease inhibitors, centrifuging for 10 min at 15,000g, and preclearing with CL-4B sepharose beads (Pharmacia). Homer affinity columns were prepared by irreversibly cross-linking Homer GST fusion protein to Affigel agarose beads (1 μ g Homer protein per 1 μ l bed volume; Bio-Rad Laboratories). 40 μ l of beads were then incubated with lysate from one hippocampus for 1 h at 4 °C, washed three times with PBS, and bound mGluR5 was eluted by boiling in $3 \times$ loading buffer. For experiments examining specificity of Homer binding to metabotropic glutamate receptors, HEK293 cells were transiently transfected with mGluR1 α , mGluR2, mGluR4 or mGluR5 expression constructs, scraped into PBS + 1% Triton, sonicated for 2×10 s, centrifuged at 15,000g for 10 min at 4 °C, and precleared. Lysate from half of a 10-cm plate was incubated with 50 μ l of beads and washed as above. Samples were analysed by western blot analysis using the appropriate polyclonal mGluR antibody. Deletion constructs of Homer were prepared by the polymerase chain reaction and cloned as fusion constructs with GST in pGEX (Pharmacia).

Coimmunoprecipitation. Hippocampal lysate was prepared as above. Rabbit anti-Homer serum or preimmune serum were irreversibly linked to Affigel agarose beads (Bio-Rad Laboratories) and washed extensively with PBS. 50 μ l beads were incubated with lysate from one hippocampus overnight at 4 °C, washed twice with PBS with 1% Triton and twice with PBS, resuspended in $3 \times$ SDS loading buffer and analysed by gel electrophoresis and western blot analysis. In control experiments, coimmunoprecipitation of mGluR5 was blocked by preincubating anti-Homer-linked beads with 50 μ g of Homer GST-fusion protein for 1 h at 4 °C.

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Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism

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The achondroplasia class of chondrodysplasias comprises the most common genetic forms of dwarfism in humans and includes achondroplasia, hypochondroplasia and thanatophoric dysplasia types I and II (TDI and TDII), which are caused by different mutations in a fibroblast growth-factor receptor FGFR3 (ref. 1). The molecular mechanism and the mediators of these FGFR3-related growth abnormalities are not known. Here we show that mutant TDII FGFR3 has a constitutive tyrosine kinase activity which can specifically activate the transcription factor Stat1 (for signal transducer and activator of transcription)^{2,3}. Furthermore, expression of TDII FGFR3 induced nuclear translocation of Stat1,

expression of the cell-cycle inhibitor p21^{WAF1/CIP1}, and growth arrest of the cell. Thus, TDII FGFR3 may use Stat1 as a mediator of growth retardation in bone development. Consistent with this, Stat1 activation and increased p21^{WAF1/CIP1} expression was found in the cartilage cells from the TDII fetus, but not in those from the normal fetus. Thus, abnormal STAT activation and p21^{WAF1/CIP1} expression by the TDII mutant receptor may be responsible for this FGFR3-related bone disease.

Fibroblast growth-factor receptors are crucial in differentiation, angiogenesis, cell migration and development⁴. TDII is a lethal form of dwarfism that results from a recurrent point mutation (lysine to glutamate at position 650; Lys650Glu) in the activation loop of the tyrosine-kinase domain of FGFR3 (ref. 5). Its phenotype is characterized by disrupted proliferation and organization of the cartilaginous growth plates of long bones⁶. Homozygous disruption of FGFR3 in mice causes severe and progressive bone dysplasia with

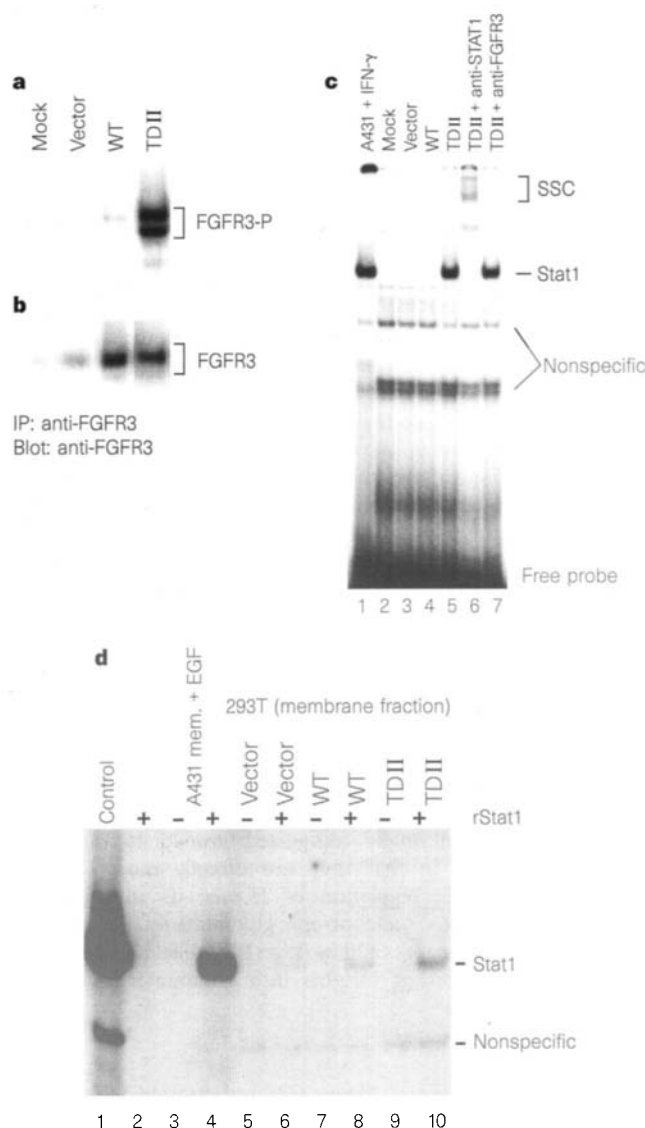


Figure 1 Activation of Stat1 by the TDII-type mutant of FGFR3. **a**, *In vitro* kinase activity of FGFR3 expressed in 293T cells. FGFR3-P, phosphorylated receptor. **b**, Relative amounts of FGFR3 proteins assayed as in **a**, assessed by immunoblotting. IP, immunoprecipitate. **c**, STAT activity in cells expressing FGFR3. The extract in lane 5 was also subjected to supershift assay with anti-Stat1 antibody (C-24; Santa Cruz) (lane 6) or with FGFR3 antibody as control (lane 7). A431 cells were stimulated with IFN-γ for 40 min (lane 1). SSC, supershifted complex. **d**, *In vitro* activation of recombinant Stat1 by the membrane fractions of 293T cells transfected with FGFR3 vectors. The same control as for **c** is shown in lane 1.

enhanced growth^{7,8}. These observations indicate that FGFR3 may inhibit cell growth in the cartilaginous growth plates, and that the disease-associated mutants have gain-of-function nature.

To study the molecular mechanism of signal transduction by wild-type and mutant FGFR3, we first assayed their kinase activities. After transient transfection into 293T cells, FGFR3 proteins were immunoprecipitated and autophosphorylated *in vitro* (Fig. 1a). The amount of wild-type and mutant FGFR3 in the precipitate was comparable (Fig. 1b), but the mutant FGFR3 had a much higher intrinsic kinase activity compared with the wild type (Fig. 1a). The double bands that were observed routinely in each gel could reflect different post-translational modification of these receptors^{9,10}.

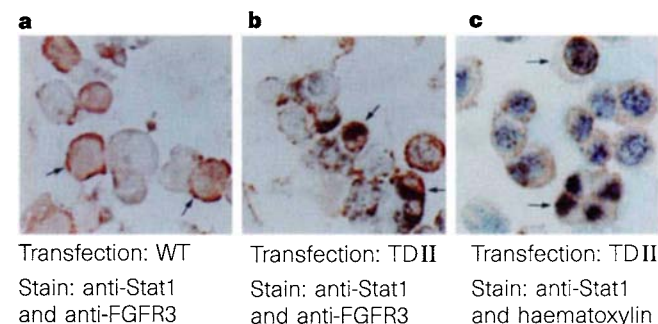


Figure 2 Translocation of Stat1 to nucleus by expression of the TDII-type mutant of FGFR3. 293T cells transfected with wild-type (a) or TDII-type (b) receptors were doubly stained with antibodies against Stat1 (black) and FGFR3 (brown). c, Anti-Stat1 stain (dark brown) of TDII-transfected cells, with counterstaining by haematoxylin showing the nucleus in blue.

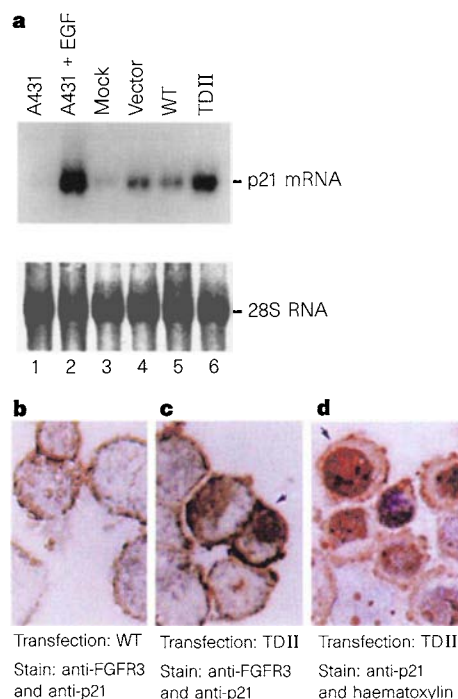


Figure 3 Increased expression of p21 as a result of expression of the TDII-type mutant of FGFR3. a, Expression of p21 mRNA is stimulated by expression of the TDII-type mutant of FGFR3 in 293T cells. RNAs from unstimulated A431 cells, and A431 cells stimulated with EGF for 8 hours¹⁸, were loaded as controls. b, c, Expression of p21 protein in 293T cells transfected with wild-type (b) or TDII-type (c; arrow) of FGFR3. Cells were doubly stained with antibodies against p21 and FGFR3 (b, c). d, Colocalization of p21 protein (brown; arrow) with haematoxylin stained nuclei (blue) in 293T cells transfected with TDII-type FGFR3.

STAT proteins can be activated by the cytokine-receptor-associated JAK tyrosine kinases and also by some receptor tyrosine kinases such as the receptor for epidermal growth factor (EGF)^{11–18}. We have previously shown that Stat1 is essential for EGF- and interferon(IFN)-induced cell-growth arrest¹⁸. We found that the expression of mutant TDII FGFR3 could also induce a protein complex that specifically bound to a high-affinity STAT-interacting site (SIE) in an electrophoretic mobility shift assay (EMSA) (Fig. 1c, lane 5). This complex comigrated with the Stat1 complex induced by IFN- γ (Fig. 1c, lane 1), and was further supershifted by a specific anti-Stat1 antibody, but not by a control antibody. These results indicate that the expression of mutant-TDII FGFR3 can activate Stat1. The wild-type receptor could also activate Stat1 when Stat1 was cotransfected with it, although the activation of Stat1 by wild-type FGFR3 was ~20-fold less than that by the TDII-mutant receptor (data not shown). The degree of Stat1 activation correlated with the constitutive kinase activities of these receptors. Moreover, Stat1 activation by the TDII-mutant FGFR3 could be enhanced by FGF1 (data not shown), a ligand for FGFR3 (ref. 19).

We tested whether FGFR3 directly mediated the activation of Stat1 by using membrane fractions containing TDII-mutant or wild-type FGFR3 and recombinant Stat1 protein *in vitro*¹⁴. As shown in Fig. 1d, neither the receptor-containing membrane fractions alone (lanes 7, 9) nor recombinant Stat1 protein alone (lane 2) had any DNA-binding activity. But the TDII membrane fraction, as well as the wild-type FGFR3 membrane fraction, could activate recombinant Stat1 in a ligand-independent fashion (Fig. 1d, lanes 8, 10); in contrast, membrane fractions from vector-transfected cells had minimal activity (lane 6), indicating that activation of Stat1 was dependent on the presence of FGFR3. Thus, FGFR3 proteins can activate Stat1 directly in the absence of ligand *in vitro*. However, the difference in the amount of Stat1 activation *in vivo* and *in vitro* suggests that (an)other factors, which might be missing from the *in vitro* system, may also contribute to Stat1 activation *in vivo* (Fig. 1c). We also used membrane fractions from A431 cells with added EGF as a positive control (Fig. 1c, lane 4), given that A431 membrane enriched for the EGF-receptor kinase activates STAT proteins only in the presence of EGF *in vitro*¹⁴.

To confirm that the receptors were expressed correctly in our transfected cell and that expression of the TDII receptor induced Stat1 activation in the cell, we investigated the intracellular localization of Stat1 associated with the expression of FGFR3. Latent Stat1 is found in the cytoplasm, but the activated protein may translocate into the nucleus^{16,20}. Translocation of Stat1 into the nucleus is therefore an indication of Stat1 activation. As shown in Fig. 2, Stat1 protein was barely evident in cells transfected with wild-type FGFR3, so was expressed at a low level, whereas FGFR3 was expressed on the cell surface (in brown in Fig. 2a, indicated by arrows). In contrast, many cells transfected with the TDII vector showed that Stat1 was concentrated in the nucleus while the TDII receptor was expressed on the membrane (Fig. 2b, arrows), suggesting that Stat1 was activated in these cells. Counterstaining of the nuclei in blue with haematoxylin over the anti-Stat1 stain confirmed the nuclear localization of Stat1 in the TDII-receptor-transfected cells (arrows in Fig. 2c).

We have previously shown that Stat1 activation induces expression of the cell-cycle inhibitor p21 (ref. 18). As expected, the level of p21 messenger RNA was raised in cells transfected with TDII receptor compared with cells transfected with other plasmids (Fig. 3a, lane 6). Probably as a result of calcium-mediated transfection, the amount of p21 mRNA was nonspecifically increased in transfected cells compared with untransfected cells (Mock, lane 3 in Fig. 3a). EGF-induced p21 mRNA in A431 cells¹⁸ served as a positive control (Fig. 3a, lanes 1, 2). Consistent with the expression of its mRNA, the amount of p21 protein was increased particularly in the nuclei of the TDII-receptor-transfected cells, as demonstrated by immunocytochemical staining with anti-p21 antibody (Fig. 3c, d,

arrows), whereas there was very little p21 in wild-type-FGFR3-transfected cells (Fig. 3b). As 293T cells express both adenovirus E1B and SV40 large-T proteins which can inactivate p53, this p21 induction by TDII receptor may not involve p53 (ref. 21).

Furthermore, the rate of DNA synthesis and the colony-formation efficiency were significantly reduced by expression of the TDII receptor, whereas the wild-type receptor did not affect either¹⁸ (Fig. 4). The growth-inhibitory effect of the TDII receptor may be more important than it appears, bearing in mind that the transfection efficiency is less than 50 per cent in these assays (Fig. 2, data not shown).

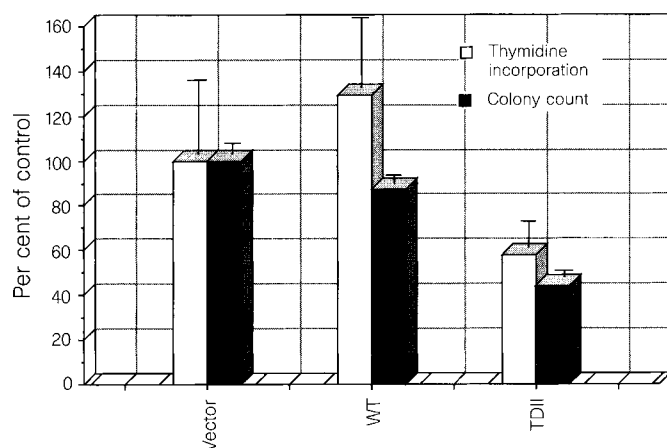


Figure 4 Growth inhibition of 293T cells expressing TDII-type mutant of FGFR3. ³H-thymidine uptake and colony formation in soft agar are shown. Each bar represents the average of 3 experiments; error bar, s.d.

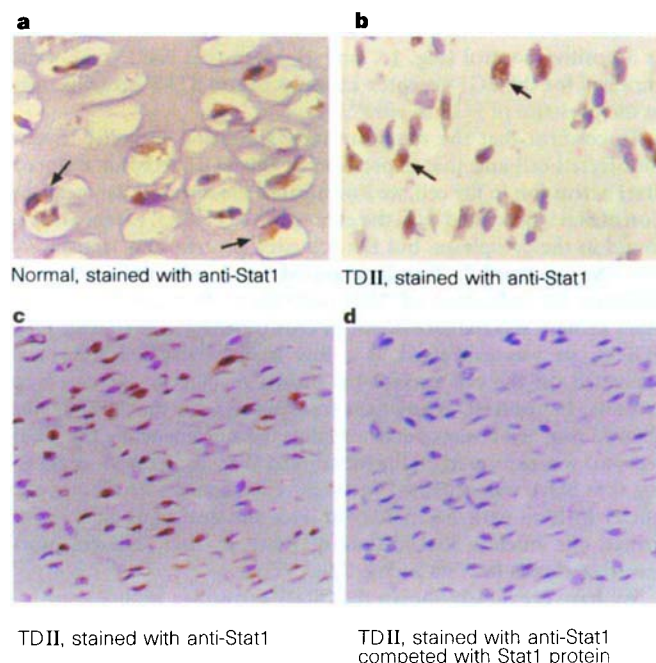


Figure 5 Stat1 activation in the chondrocytes of TDII patients. **a**, Section of bone from a normal fetus (19 weeks gestation) showing the cytoplasmic stain of Stat1 (arrow). The brown colour (Stat1 staining) in the cytoplasm (arrows) is separate from the blue-purple-stained nucleus. **b**, Section of bone from a TDII fetus. Stat1 (brown) is exclusively stained in the nucleus (arrow). **c**, Brown staining (Stat1) in the nucleus in TDII chondrocytes overlaps the blue-purple-stained nucleus to generate the dark-brown colour. **d**, When Stat1 is added as a competitor, the nuclear Stat1 staining is almost all removed (no brown colour), leaving the blue-purple nucleus that was counterstained by haematoxylin.

Our results indicated that Stat1 activation and p21 induction might together be responsible for the TDII receptor-induced developmental defect. We therefore tested chondrocytes in growth plates from TDII-affected individuals and control individuals for Stat1 activation and p21 expression by using immunohistochemical methods. Heterozygosity for the Lys650Glu mutation had been confirmed in two of the TDII patients (data not shown). As shown in Fig. 5, there was a small amount of Stat1 in the cytoplasm of chondrocytes from femur and rib of a normal subject (Fig. 5a: Stat is brown stained (arrows), nuclei are counterstained in blue), indicating that Stat1 is not activated in normal cells. However, Stat1 stained exclusively in the nuclei of many chondrocytes at the growth plates of the femur (Fig. 5b, arrows) and of vertebrae (data not shown) from three TDII-affected individuals, indicating that Stat1 was activated in TDII chondrocytes. This nuclear staining by anti-Stat1 antibody in chondrocytes of a TDII-affected patient (Fig. 5c) was abolished in the presence of recombinant Stat1 protein as a competitor (Fig. 5d), confirming that this anti-Stat1 antibody specifically recognizes Stat1 protein. The area of nuclear Stat1 staining in the growth plate corresponded to that of intense staining by anti-FGFR3 antibody (data not shown), suggesting that Stat1 translocation was triggered by the mutant TDII receptor.

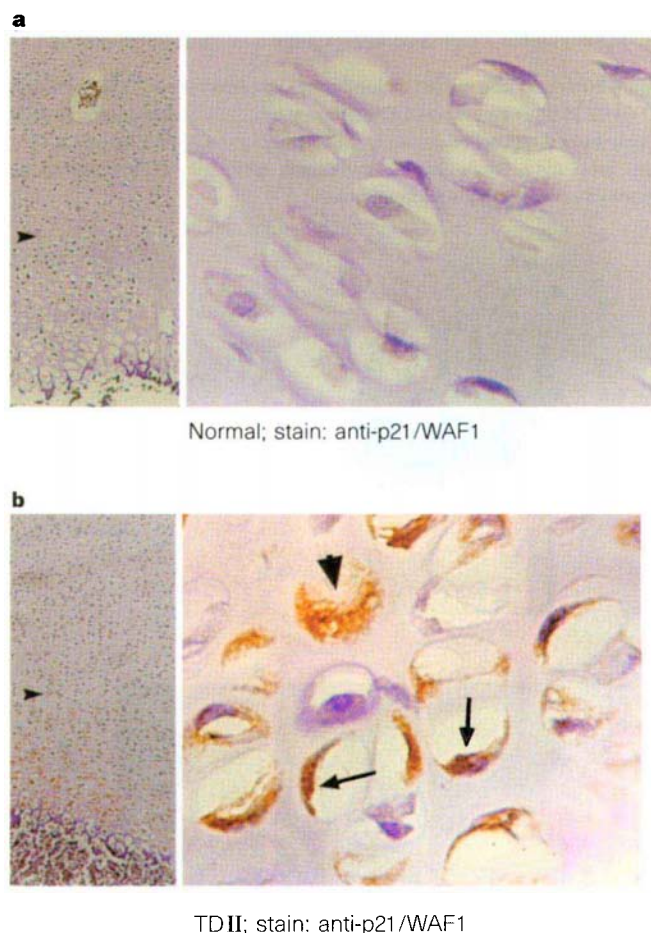


Figure 6 Increased expression of p21 in the chondrocytes of TDII patients. Each panel shows low ($\times 80$, left) and high ($\times 1,200$, right) magnifications. Arrowheads in the low-magnification view indicate the areas shown at higher magnification. **a**, Section of bone from the normal fetus. **b**, Section of bone from a TDII patient: the expression of p21 protein is demonstrated by brown staining in the nuclei (arrow). Arrowhead, a degraded cell (see text).

Figure 6a shows that no p21 protein was present (no brown stain) in chondrocytes from a normal individual. However, TDII chondrocytes did express p21 (dark brown nuclear staining by anti-p21 antibodies; Fig. 6b, arrows). The p21 staining is mostly in the nucleus, but was evident in the cytoplasm in some cells (Fig. 6b, arrowhead). The presence of p21 may retard growth of TDII chondrocytes, resulting in anomalous bone development. There were also vacuole-like structures in these cells, which may be indicative of the cell degeneration frequently observed in TDII chondrocytes.

FGFRs may initiate mitogenic pathway, possibly involving Ras protein, MAP kinases or other signalling proteins^{22,23}, and the STAT signalling pathway simultaneously, which may negatively regulate cell growth by inducing cell-cycle inhibitors such as p21. In normal development, STAT activation by FGFRs may be required for proper negative control to balance the actions of the mitogenic pathway, so cells can grow or differentiate under the influence of both pathways. The abnormally high kinase activity of the TDII-mutant receptor (Fig. 1a, b)^{9,10} caused specific Stat1 activation, p21 induction and cell-growth arrest in chondrocytes and other cells expressing FGFR3 (ref. 24), disrupting normal growth and development. The intensity of signalling by the mutant TDII receptor can therefore have very different cellular effects²⁵. We found no significant difference in the state of phosphorylation of MAP kinases in 293T cells transfected with either the TDII or other vectors (our unpublished results). Stat1 null mice in the early studies showed no overt defect in their development^{26,27}, which might be due to redundancy in the STAT family proteins or to the possibility that Stat1 can only be activated when FGFR3 has the constitutively activating mutation. The mutant receptors responsible for achondroplasia and hypochondroplasia weakly activated Stat1 in our transfection assay, which may correlate with the milder phenotypes of these chondrodysplasias compared with TDII (unpublished result). To our knowledge, this is the first example of a human disease that involves an abnormality in the STAT pathway. It will be interesting to see whether other FGFR-associated diseases¹ involve STAT activation and whether interference with this activation or with p21 function might provide a basis for treating the different forms of dwarfism. □

Methods

Site-directed mutagenesis and plasmid construction. A Chameleon double-stranded, site-directed mutagenesis kit (Stratagene) was used to engineer the TDII-type mutation into the mouse FGFR3 expression vector pMo/mFR3/SV¹⁹, together with an oligonucleotide (5'-GGACTACTACAAG-GAGACCACAAACGGCCGGCTACC-3') (K644E in mouse) and an AflIII primer from the manufacturer; successful mutagenesis was verified by sequencing. The mutant and wild-type cDNAs cloned into pEF-BOS vector²⁸ were used for transfections unless otherwise indicated.

In vitro kinase assay of FGFR3. 293T cells were transfected with each expression vector (15 µg, pMo/SV) by using calcium phosphate precipitation. Two days after transfection, whole-cell extracts were prepared, and FGFR3 was immunoprecipitated with an anti-FGFR3 antibody (Santa Cruz). An aliquot of the immunoprecipitate was incubated in a kinase buffer containing [γ -³²P]ATP as described²⁹. After washing, products were analysed by 6% SDS-PAGE and autoradiography. An aliquot of the immunoprecipitate was also immunoblotted with anti-FGFR3 antibody and chemiluminescence used for development (ECL, Amersham).

Analysis of STAT activation. Whole-cell extracts were prepared from 293T cells 2 days after transfection with each expression vector. They were subjected to EMSA using ³²P-labelled m67 as a probe¹⁸. Equivalent amounts (10 µg) of protein were applied to each lane.

Protein expression and purification. Recombinant human Stat1 protein, with a hexahistidine tag at its N terminus, was synthesized using the Xpress baculovirus expression system (Invitrogen) with insect SF9 cells. Cells were lysed and proteins purified by affinity chromatography on ProBond nickel resin (Invitrogen) according to the manufacturer's instructions (native condition).

Fractions containing Stat1, as detected by immunoblotting, were dialysed against hypotonic buffer containing 120 mM NaCl and 10% glycerol¹⁴.

In vitro activation of Stat1 protein. Membrane fractions from A431 cells or 293T cells that had been transfected with each expression vector were prepared as described¹⁴. In vitro activation of recombinant Stat1 protein by these membrane fractions was done as described¹⁴, except that the cytoplasm was replaced by recombinant Stat1. After reaction, aliquots were subjected to EMSA using ³²P-labelled m67 as probe¹⁸.

Immunocytochemistry and immunohistochemistry. Two days after transfection, 293T cells were collected and smeared on glass slides. Cells were fixed and permeabilized in cold methanol/acetone (1:1) solution at -20°C for 10 min, followed by incubation with normal horse or goat serum to block nonspecific binding sites. Paraffin sections were deparaffinized by successive treatment with xylene, 95% ethanol and water. After treatment with FICIN (Zymed) enzyme to retrieve antigens, sections were incubated in 2.0% hydrogen peroxide and 0.1% sodium azide in methanol for 10 min to inactivate endogenous peroxidase. Antibodies against Stat1 (monoclonal for cultured cells and polyclonal for tissue sections; Transduction Labs), p21^{WAF1/CIP1} (187; Santa Cruz), and FGFR3 (Santa Cruz), together with a Vectastain Elite ABC Kit (Vector Labs), were used to stain the cells. 3,3'-Diaminobenzidine with or without nickel chloride was used as substrate for peroxidase to give a black or brown colour, respectively³⁰.

Northern blotting. Total RNAs (5 µg) from 293T cells transfected with the FGFR3 expression vectors were subjected to northern blot analysis with p21 cDNA as probe¹⁸.

Growth assays. Twenty-four hours after transfection, 293T cells were replated into 24-well culture plates at a density of 1.5×10^4 cells per well and cultured for an additional 24 hours. Cells were then labelled with ³H-thymidine ($1.5 \mu\text{Ci ml}^{-1}$) for 4 h, washed twice with PBS and three times with ice-cold 10% trichloroacetic acid. Incorporated radioactivity was extracted by incubation in 3% perchloric acid at 95°C for 40 min and measured by liquid scintillation. 293T cells were used for clonogenic assay after transient transfection as described¹⁸, except that cells was reduced as 5×10^3 cells per plate. Colonies were counted 10 d after plating.

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A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection

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Macrophage type-I and type-II class-A scavenger receptors (MSR-A) are implicated in the pathological deposition of cholesterol during atherogenesis as a result of receptor-mediated uptake of modified low-density lipoproteins (mLDL)^{1–6}. MSR-A can bind an extraordinarily wide range of ligands, including bacterial pathogens⁷, and also mediates cation-independent macrophage

adhesion *in vitro*⁸. Here we show that targeted disruption of the MSR-A gene in mice results in a reduction in the size of atherosclerotic lesions in an animal deficient in apolipoprotein E. Macrophages from MSR-A-deficient mice show a marked decrease in mLDL uptake *in vitro*, whereas mLDL clearance from plasma occurs at a normal rate, indicating that there may be alternative mechanisms for removing mLDL from the circulation. In addition, MSR-A-knockout mice show an increased susceptibility to infection with *Listeria monocytogenes* or herpes simplex virus type-1, indicating that MSR-A may play a part in host defence against pathogens.

Mice deficient in type I and type II MSR-A were generated by disrupting exon 4 of the MSR-A gene, which is essential for the formation of functional trimeric receptors (Fig. 1a). Mice heterozygous and homozygous for the MSR-A mutation were normal in both appearance and growth and were fertile. MSR-A deficiency was transmitted in a mendelian fashion, as identified by Southern blot analysis of tail DNA (Fig. 1b). Immunostaining of liver sections from wild-type animals, using anti-MSR-A monoclonal antibody 2F8 (ref. 8), detected MSR-A protein in sinusoidal cells^{6,9,10} (Fig. 1c). In homozygous MSR-A-deficient mice (MSR^{-/-}), no immunoreactive protein was detected (Fig. 1d). 2F8 can bind to both type I and type II MSR-A, indicating that MSR^{-/-} mice are deficient in both type I and II receptor proteins.

We measured the rate of degradation of modified LDL, in the form of acetylated LDL (acetyl-LDL) or oxidized LDL, by thioglycollate-elicited peritoneal macrophages, a convenient source of cells recruited by an inflammatory stimulus. Saturable high-affinity degradation of acetyl-LDL was reduced to less than a third in cultured macrophages from MSR^{-/-} mice (Fig. 2a). Oxidized LDL degradation activity from MSR^{-/-} animals was also reduced, but ~50% of the activity still remained (Fig. 2b). Furthermore, there was a striking decrease in the specific cell association and in the degradation of bovine serum albumin modified by advanced-glycation (advanced-glycation end products), AGE-BSA (Fig. 2c). This indicates that MSR-A may be more important in the metabolism of AGE proteins in these cells than other putative AGE receptors¹¹.

To assess the role of the MSR-A in mLDL clearance from the circulation, we measured the rate of removal of acetyl-LDL from plasma. Surprisingly, there was no apparent difference in clearance rate after injection of low (5 μ g) or high (200 μ g) amounts of ¹²⁵I-labelled acetyl-LDL (Fig. 2d). Trichloroacetic-acid-precipitable counts in serum did not differ between genotypes. Liver uptake values at 5 min after injection were $77.9 \pm 7.3\%$ and $75.2 \pm 8.9\%$ of injected dose in MSR^{+/+} and MSR^{-/-} mice, respectively. These results suggest that, in contrast to our observations with cultured macrophages, in the liver there are receptors other than MSR-A, for example type B and/or C scavenger receptors, which can mediate the uptake of mLDL. There were no significant differences in plasma cholesterol (147.39 ± 33.75 mg dl⁻¹ compared with 142.81 ± 33.55 mg dl⁻¹ for MSR^{-/-} and MSR^{+/+}, respectively), triglyceride (245.38 ± 88.56 mg dl⁻¹ versus 255.10 ± 47.63 mg dl⁻¹ for MSR^{-/-} and MSR^{+/+}, respectively) or lipoprotein composition (not shown) between normal and MSR^{-/-} mice. After feeding on a high-fat diet containing 1.25% cholesterol for 11 weeks, the plasma cholesterol and triglyceride levels of MSR^{-/-} mice were 312.0 ± 86.02 and 68.9 ± 21.84 mg dl⁻¹, which are not significantly different from those of normal mice.

Peritoneal macrophages from MSR^{-/-} mice were able to adhere to a tissue-culture plastic vessel or a glass surface, but the process was slower than for normal macrophages. As shown in Fig. 3a and b, 24 hours after addition to glass more than half of the wild-type macrophages were tightly attached, with well spread cytoplasm. By contrast, most macrophages from MSR^{-/-} still remained rounded and unspread. It took nearly 40 hours for macrophages from MSR^{-/-} mice to adhere and spread like wild-type macrophages.

In the presence of 5 mM EDTA, wild-type macrophages could adhere to a plastic vessel, but most MSR^{-/-} macrophages could not (Fig. 3c). These results confirm that MSR-A is essential as a macrophage adhesion molecule *in vitro*. Hepatic granulomas induced by the injection of particle glucans in MSR^{-/-} mice were larger, more diffuse and less circumscribed compared with those in MSR^{+/+} mice. These features of MSR^{-/-} mice may have been

caused by the deficiency of MSR-A as an adhesion molecule on macrophages.

To clarify the effect of MSR-A deficiency on the progression of atherosclerosis, we adopted a 'double knockout' strategy^{12,13}. MSR-A-deficient mice were crossed with apoE-deficient mice (apoE^{-/-}), which have high plasma cholesterol and develop severe atherosclerotic lesions¹⁴⁻¹⁶. Double-knockout mice (MSR-A/apoE) were

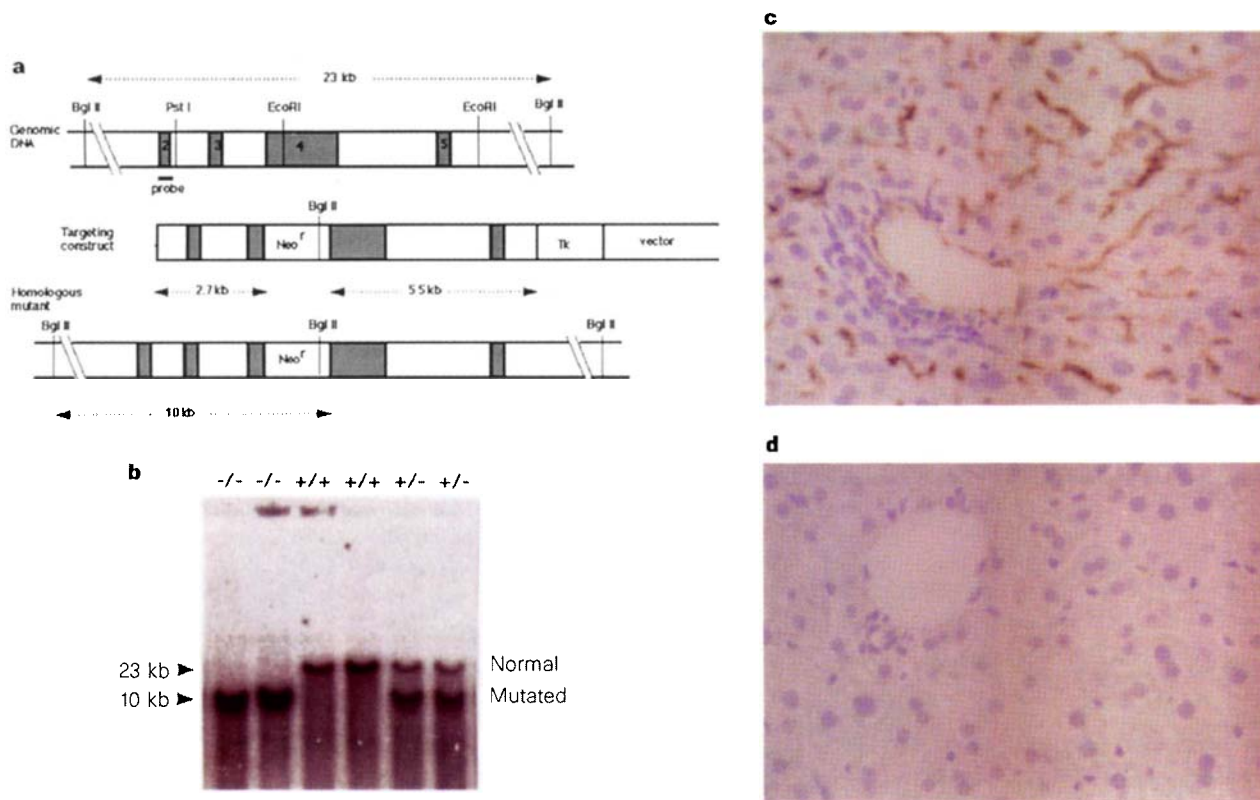


Figure 1 Targeting of the mouse MSR gene. **a**, The targeting construct of the mouse MSR gene and predicted structure of the targeted MSR locus. **b**, Southern blot analysis of BglII-digested genomic DNA. The 10-kb BglII fragment represents the homologous recombinant allele. **c** and **d**, Frozen sections of liver

immunostained with anti-murine MSR-A monoclonal antibody⁸ 2F8 for MSR-A. In normal (MSR^{+/+}) liver, MSR-A protein is detected in sinusoidal cells and Kupffer cells (**c**), whereas no staining is seen in MSR^{-/-} liver (**d**). Tissues are counter-stained with haematoxylin.

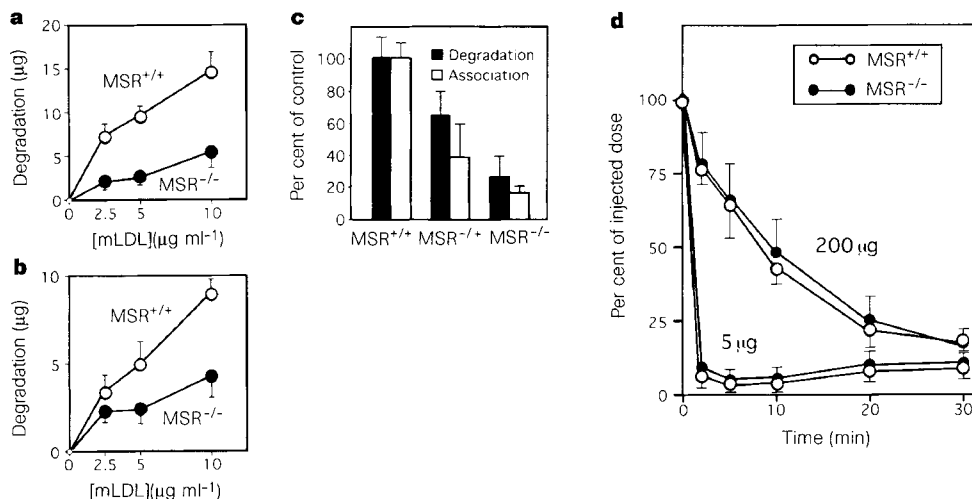


Figure 2 Modified-lipoprotein (mLDL) metabolism in MSR^{-/-} compared with MSR^{+/+} mice. **a**, Degradation of ¹²⁵I-labelled acetyl-LDL; **b**, ¹²⁵I-labelled oxidized-LDL; and **c**, ¹²⁵I-labelled AGE-BSA by peritoneal macrophages. Cell association is also shown in **c**. Degradation is given as micrograms of protein degraded per mg of protein in 5 h for **a** and **b**, and 18 h for **c**. **d**, Clearance of intravenously injected

¹²⁵I-acetyl-LDL from plasma of MSR^{-/-} (*n* = 3) and MSR^{+/+} (*n* = 3) mice. After injection of radiolabelled acetyl-LDL (5 or 200 µg protein), 50 µl blood was taken from the inferior vena cava at the indicated times and counted²⁸. Data are presented as means ± s.d.

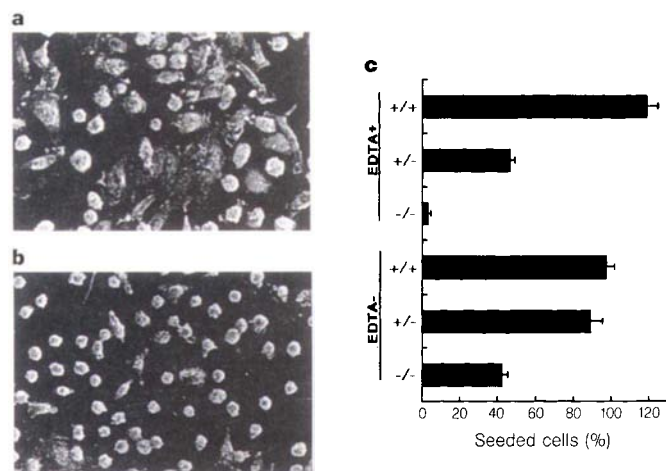


Figure 3 Adhesion properties of MSR-deficient macrophages. **a, b**, Scanning electron micrographs of peritoneal macrophages from MSR^{+/+} (**a**) and MSR^{-/-} (**b**) mice after 24 h in culture. About 60% of MSR^{+/+} mouse macrophages adhere tightly to glass, with their cytoplasm and plasma membrane spreading well (**a**). In contrast, more than 85% of macrophages from MSR^{-/-} mice still remain round, with few cytoplasmic processes (**b**). **c**, Adhesion of MSR-deficient macrophages to tissue-culture-treated plastic surfaces. Data are means $\pm$ s.d.

fertile and appeared to be healthy. Body, liver and spleen weights did not differ significantly between double- and single-knockout mice (data not shown). Plasma cholesterol in MSR-A/apoE double-knockout mice (980.3 ± 313.7 mg dl⁻¹) was significantly higher than that of apoE^{-/-} mice (670.2 ± 221.4 mg dl⁻¹; $P = 0.005$). However, as shown in Fig. 4, the average (arithmetic mean) size of the atherosclerotic lesions in MSR-A/apoE double-knockout mice ($10.5 \pm 7.14 \times 10^4 \mu\text{m}^2$) was significantly smaller than that of apoE single-knockout littermates ($24.9 \pm 15.5 \times 10^4 \mu\text{m}^2$; $P = 0.003$). There was no correlation between lesion size and plasma cholesterol concentration in either the apoE^{-/-} or MSR-A/apoE double-knockout mice. The lesions from both the apoE^{-/-} and MSR-A/apoE double-knockout mice consisted mainly of foamy macrophages, with occasional intimal smooth-muscle cells. Macrophages in apoE^{-/-} lesions stained positive with the anti-mouse MSR-A monoclonal antibodies 2F8 and with the anti-mouse macrophage (CD68) antibody FA11 (ref. 17), whereas macrophages in double-knockout mice were positive for FA11, but negative for 2F8 staining. These results are direct evidence that MSR-A plays a role in the development of atherosclerosis *in vivo*. MSR-A deficiency causes a decrease in mLDL uptake by macrophages *in vitro*, but does not change the clearance rate of mLDL from plasma. This suggests that modification of LDL in the arterial wall and successive uptake by macrophages is more important for lesion development than the uptake of mLDL directly from the plasma. The continuing presence of lesions in MSR-A/apoE double-knockout mice and foam cell formation in MSR-A-negative macrophages indicates that other scavenger receptors, such as MARCO¹⁸, macrophage mannose receptor (CD11b), CD36 (ref. 20) and SR-B1 (ref. 21), may also participate.

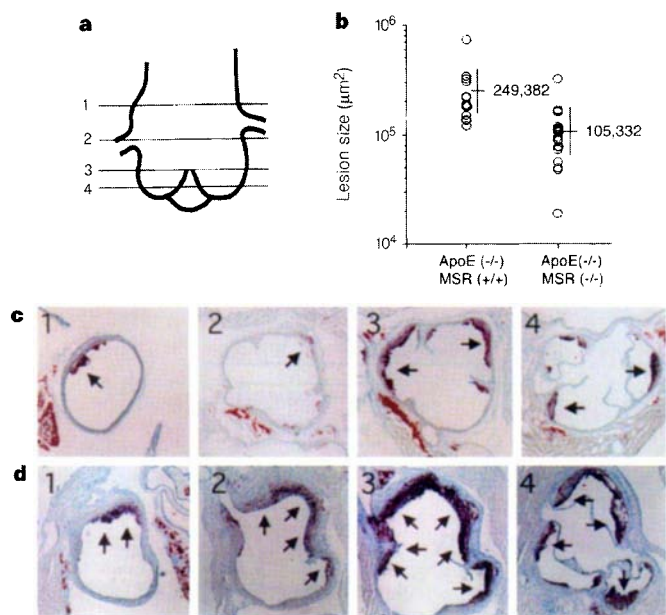


Figure 4 Evaluation of atherosclerotic lesions in apoE-knockout and MSR-A-and-apoE double-knockout mice. **a**, The four sections shown were used for quantitative evaluation of atherosclerotic lesions¹⁶: (1) the most proximal part of the ascending aorta had a round cross-section; (2) The valve attachment sites and the coronary ostia, but not the valve leaflets; (3) the valve leaflets appear as small nodules in the valve attachment sites; (4) the valves appear complete and are joined to their attachment sites. **b**, The average size of the atherosclerotic lesions in sections 1-4 in apoE-knockout ($n = 14$) and MSR-A/apoE double-knockout ($n = 15$) females at 5 months of age. Statistical analysis used Student's *t*-test. **c** and **d**, Representative sections from MSR-A/apoE double-knockout (**c**, 1-4) and apoE knockout mice (**d**, 1-4). Arrows indicate atherosclerotic lesions.

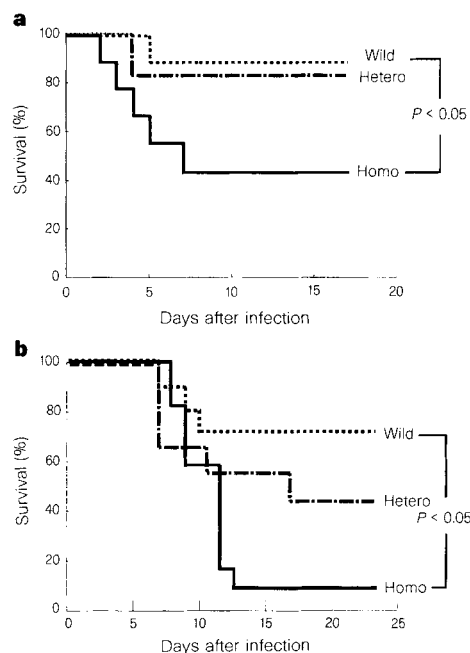


Figure 5 Survival of MSR-A-deficient mice after infection with pathogens. **a**, Survival after *L. monocytogenes* infection (10^6 CFU per mouse). **b**, Survival after HSV-1 infection (5×10^5 PFU per mouse). For statistical analysis, the Wilcoxon method was used.

Table 1 Multiplication of *L. monocytogenes* in MSR-A-knockout mice

Genotype		Numbers (log) of bacteria (mean $\pm$ s.d.)						
		10 min	1 h	6 h	24 h	48 h	96 h	144 h
Liver	-/-	3.8 $\pm$ 0.05	3.1 $\pm$ 0.37	2.0 $\pm$ 0.61	4.5 $\pm$ 0.30	4.5 $\pm$ 0.82	4.2 $\pm$ 1.28*	1.5 $\pm$ 1.41
	+/+	4.0 $\pm$ 0.06	3.0 $\pm$ 0.19	2.2 $\pm$ 0.19	3.7 $\pm$ 0.33	4.2 $\pm$ 0.71	2.4 $\pm$ 0.29*	1.1 $\pm$ 0.99
Spleen	-/-	4.3 $\pm$ 0.10	3.6 $\pm$ 0.20	4.3 $\pm$ 0.45	5.9 $\pm$ 0.12*	5.9 $\pm$ 0.52	5.5 $\pm$ 0.54	2.6 $\pm$ 1.06
	+/+	4.1 $\pm$ 0.33	3.4 $\pm$ 0.29	3.9 $\pm$ 0.32	5.4 $\pm$ 0.02*	5.3 $\pm$ 0.23	3.8 $\pm$ 0.39	2.6 $\pm$ 1.56
Blood	-/-	1.8 $\pm$ 0.08	0	0.3 $\pm$ 0.24	0.1 $\pm$ 0.17	0.2 $\pm$ 0.39	0.2 $\pm$ 0.35	0
	+/+	2.2 $\pm$ 0.14	0.1 $\pm$ 0.17	0	0	0	0	0

Mice were each infected with 10^5 CFU *L. monocytogenes*.
* $P < 0.05$.

To clarify the role of MSR-A in host defence, MSR^{-/-} mice were infected with macrophage-tropic microorganisms and their survival monitored. When a lethal dose of *L. monocytogenes* was injected, the survival of MSR^{-/-} mice was significantly lower than for wild-type mice (Fig. 5a). The injected bacteria rapidly disappeared from the circulation in animals of each genotype. The multiplication of *L. monocytogenes* in MSR^{-/-} spleen and liver was significantly increased 24 h and 96 h after injection compared with wild type. The multiplication of *L. monocytogenes* in the liver in the period between 10 min and 6 h after injection was similar to wild type (Table 1). These results indicate that the susceptibility of MSR^{-/-} mice may be related to a defect in the uptake or killing of bacteria by macrophages, and not to the clearance of bacteria from the plasma. Furthermore, when 5×10^5 PFU of the herpes simplex virus (HSV-1) were injected intraperitoneally into MSR^{-/-} animals, survival was significantly lower than for wild-type mice (Fig. 5b).

In conclusion, we have quantified the role of MSR-A *in vivo* in the clearance of mLDL. MSR-A plays a part in macrophage adherence and helps the macrophage to combat infectious pathogens. We have shown that MSR-A contributes to the generation of atherosclerotic lesions *in vivo*. □

Methods

Generation of MSR-A-knockout mice. A targeting vector was constructed from an 8.2-kb MSR-A genomic fragment encompassing exons 3 to 5. We inserted a 1.8-kb *XhoI*-*SacI* fragment of pGK1neo-poly(A)⁺ into the *EcoRI* site in exon 4, which encodes the α -helical coiled-coil structure of both the type I and type II trimeric receptors, and flanked a 1.8-kb *XhoI*-*SacI* fragment of the HSV-tk gene downstream of the MSR-A sequence. A3-1 ES cells²² were transfected by electroporation with a linearized targeting vector. G418/gancyclovir-resistant clones were screened and ES cells containing the disrupted allele were injected into C57BL/6J (CLEA, Japan) blastocytes as described²³. Embryos were transferred into the uteri of ICR (CLEA, Japan) recipients as described²⁴. To obtain heterozygous mutants, chimaeras were mated with ICR females. Brother-sister mating of heterozygotes was used to generate homozygous mutants.

Degradation and cell-association assay in MSR-A-deficient macrophages. Three days after intraperitoneal injection of thioglycollate broth (Difco), peritoneal macrophages were collected from each genotype animal²⁵ and were seeded on a 24-well plate at a density of 3×10^5 cells per well. For oxidation of LDL, 200 μ g ml⁻¹ LDL in Ham F10 medium containing 10 μ M CuSO₄ were incubated at 37°C for 20 h. Degradation of m-LDL was assayed as described². AGE-BSA degradation and cell-associated activity of peritoneal macrophages were measured as before²⁶. AGE modification was achieved by incubating 2 g BSA in 10 ml 0.5 M sodium phosphate buffer, pH 7.4, with 3 g D-glucose at 37°C for 40 weeks.

Adhesion of MSR-A-deficient macrophages to substrate. Thioglycollate-induced peritoneal macrophages were plated onto glass slides in a culture dish (35 mm; Nunc) at 0.5×10^6 cells ml⁻¹. After 24 h of culture at 37°C in RPMI 1640 supplemented with 10% FCS, the macrophages were observed on glass slides using a JEOL scanning electron microscope. Thioglycollate-induced peritoneal macrophages were also incubated in RPMI-1640 supplemented with 10% FCS at a density of 1×10^5 cells per well in a 96-well plate (Falcon) for 30 min at 37°C, in 5% CO₂ with air. EDTA was added to a final concentration of 5 mM where indicated. Cells were fixed in methanol for

staining and quantification. Briefly, after staining with 10% (v/v) Giemsa solution for 20 min, plates were washed with tap water, dried, and the retained dye solubilized in methanol. Staining was measured as absorbance at 450 nm using an automatic plate reader (Titertek Multiskan; MCC/340, Flow Laboratories).

Evaluation of atherosclerotic lesions. To obtain knockout mice homozygous for disruption of both the MSR and apoE loci, apoE-knockout mice¹⁴ were mated with MSR-A-knockout mice. The offspring, which were heterozygous for disruption of both MSR-A and apoE, were bred with each other to produce MSR-A/apoE double-knockout mice and apoE-knockout mice as a positive control. Animals were fed with normal chow (CE-2, CLEA, Japan). For the quantitative evaluation of atherosclerotic lesions, four sections of 10 μ m thickness were selected and evaluated morphometrically according to ref. 15 (Fig. 4a). Each frozen section was stained with Oil Red O and counterstained with haematoxylin. Atherosclerotic lesions were measured by NIH image 1.59 (public domain software). The average size of the lesions in the four sections was used to represent the lesion size for each mouse.

Infection with pathogens in MSR-A knockout mice. *L. monocytogenes* (serotype 4b), which produces a lethal infection in normal mice, was injected intravenously into 6–9 animals of each genotype (10^6 CFU per mouse). Their survival was assessed daily for 18 days. To assess the multiplication of *L. monocytogenes* after infection (10^5 CFU per mouse), the organs of infected mice were removed aseptically, weighed and homogenized in sterile saline. Serial dilutions of homogenized tissue or blood (0.1 ml) with PBS were plated on brain-heart infusion agar (Difco), and CFU were counted after incubation for 16 h at 37°C. In addition, 9–12 mice of each genotype were inoculated with 5×10^5 PFU of HSV-1 (Miyama +GC strain) intraperitoneally. Their survival was noted daily for 25 days. HSV-1 was passaged several times in green-monkey kidney cells and titrated as described²⁷.

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Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation

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For cells of the innate immune system to mount a host defence response to infection, they must recognize products of microbial pathogens such as lipopolysaccharide (LPS), the endotoxin secreted by Gram-negative bacteria¹. These cellular responses require intracellular signalling pathways, such as the four MAP kinase (MAPK) pathways^{2–6}. In mammalian cells the MAPK p38 is thought to play an important role in the regulation of cellular responses during infection through its effects on the expression of proinflammatory molecules^{7–9}. One means of understanding the role of p38 in these responses is to identify proteins with functions regulated by p38-catalysed phosphorylation. Here we demonstrate a link between the p38 pathway and a member of the myocyte-enhancer factor 2 (MEF2) group of transcription factors. We found that in monocytic cells, LPS increases the transactivation activity of MEF2C^{10–12} through p38-catalysed phosphorylation. One consequence of MEF2C activation is increased *c-jun* gene transcription. Our results show that p38 may influence host defence and inflammation by maintaining the balance of *c-Jun* protein consumed during infection.

We used the yeast two-hybrid system to identify proteins that interact with p38. Because active kinase–substrate interactions may be transient, we used a kinase-inactive mutant of p38 to favour the formation of stable protein–protein complexes⁵. We used as targets two constructs of p38 that were produced by fusing the cDNA of the p38 coding region (wtp38) or a p38 mutant with the TGY dual phosphorylation site mutated to AGF (TY mutant) to the carboxy

terminus of the GAL4 DNA-binding domain in the pAS1 vector¹³. A human fetal brain cDNA library fused with the GAL4 activation domain placed in the pGAD10 was screened using the two p38 constructs. By screening 10⁷ transformants we identified five positive clones which interacted with the TY mutant; in contrast, no clones were obtained when wtp38 was used in the screen. To address the specificity of these interactions a mating experiment was performed in which the cDNAs from the five clones were transfected into strain Y190. The resultant cells were mated with strain Y187 transfected with seven different cDNAs (Fig. 1a) fused with the GAL4 DNA binding domain in the pAS1 vector. Only the cells expressing the p38 TY mutant and the five test clones displayed positive galactosidase activity. Sequencing showed that each clone encoded different regions of the same protein, namely, myocyte-enhancer factor 2C (MEF2C), a transcription factor belonging to the MADS superfamily^{10–12}. MEF2C has three alternatively spliced exons of which the first was found in all our clones while alternatively spliced exons two or three were absent in other clones (Fig. 1b). The MEF2C used for subsequent experiments lacks alternatively spliced exon three. There are four members of MEF2 gene family, MEF2A–D¹⁴, but the interaction with p38 seems to be selective for MEF2C, as no other members of this family have been isolated by two-hybrid screening even though the cDNAs encoding these other proteins are in the brain library (data not shown).

To show that wtp38 and MEF2C interact, glutathione-agarose, with or without bound recombinant GST–wtp38, was incubated with varying amounts of recombinant MEF2C in the presence or absence of ATP. Immunoblotting revealed saturable binding of MEF2C to GST–wtp38-agarose but not to GST–agarose (Fig. 1c, bottom). The binding of MEF2C to p38 was not detected when a large excess of cold ATP was included (Fig. 1c, bottom, last lane). Phosphorylation of MEF2C was detected by the inclusion of ³²P-ATP in the incubation mixtures (results not shown). Taken together these data show that MEF2C and wtp38 interact, and that the stability of this interaction is reduced after phosphorylation of MEF2C.

To determine whether other MAPKs can phosphorylate MEF2C we compared the activity of recombinant p38 and two other MAP kinase family members, ERK2 and JNK1, towards MEF2C (Fig. 1d).

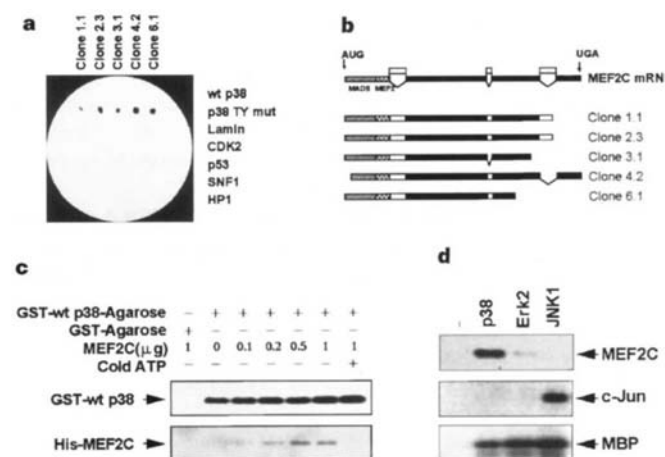


Figure 1 The MAP kinase p38 interacts with MEF2C. **a**, Clones isolated from the yeast two-hybrid screen interact selectively with the p38 TY mutant. Positive interactions are indicated by β -gal reporter activity of lifted yeast clones, shown as dark spots. **b**, The five isolated clones all encode MEF2C; schematic diagrams of the sequence of each clone are shown compared with that of full-length MEF2C cDNA. The boundaries of the MADS and MEF2 boxes are indicated; alternatively spliced regions are shown by open boxes. **c**, MEF2C binds to wtp38 *in vitro*. **d**, MEF2C is phosphorylated selectively by p38 but not by other MAP kinases.

MEF2C is indeed a preferred substrate of p38; not unexpectedly, other substrate preferences were observed for ERK2 or JNK1 (Fig. 1d). Quantification of MEF2C phosphorylation showed that p38 is 16-fold more active than ERK2 and 100-fold more active than JNK1; the relative enzyme activities were normalized using MBP.

MEF2C transcripts have previously been reported to be enriched in skeletal muscle, spleen and brain¹⁰⁻¹². By using RNase protection assays with a MEF2C probe corresponding to bases 404-695, we observed mRNA expression in primary monocytes, in monocyte-like cell lines THP-1 and U937, and in the B-cell line Akata, but not in HepG2 cells (Fig. 2a). MEF2C has been reported to bind to AT-rich DNA sequences present in promoters of a variety of genes¹⁰⁻¹². To evaluate whether activation of monocytic cells with LPS influences proteins in nuclear extracts that bind to a MEF2 site, an electrophoretic mobility shift assay was performed using oligonu-

cleotides encoding a MEF2 binding site¹¹. Treating RAW 264.7 cells with LPS resulted in a time-dependent increase in MEF2-site binding activity, reaching a maximum 1 h after stimulation with LPS (Fig. 2b). This binding activity can be competed with the oligonucleotide containing a MEF2 site but not a mutated MEF2 site.

To investigate further how p38 regulates MEF2C we used a modified form of MEF2C in which the MADS and MEF2 domains were deleted, with the remainder of the protein containing a transactivation domain¹⁵ fused to the DNA-binding domain of yeast transcription factor GAL4. This fusion protein will bind to GAL4 DNA-binding sites and can be used to study interactions between p38 and MEF2C without complications from the effects of other endogenous proteins. To assess the activation of the GAL4-MEF2C protein, RAW 264.7 or THP-1 cells were co-transfected with a

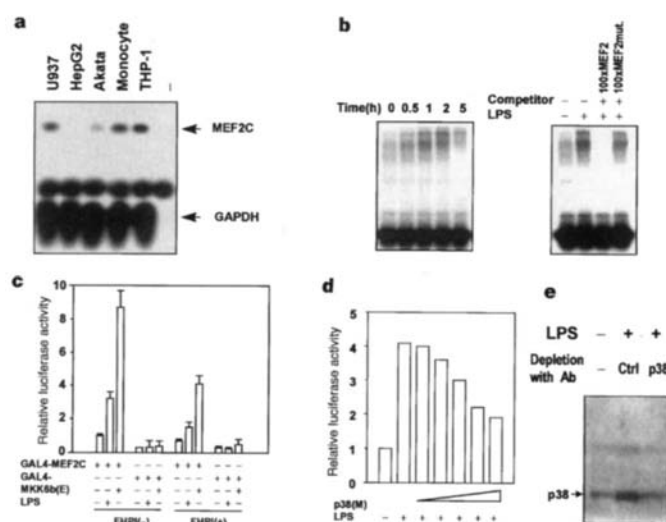


Figure 2 Investigation of MEF2C in myeloid lineage cells. **a**, RNase protection experiments show that MEF2C mRNA is present in myeloid lineage cells and cell lines. **b**, LPS induces MEF2-site binding activity in nuclear extracts of the murine cell line RAW 264.7. **c**, LPS treatment or expressions of MKK6b(E) increases MEF2C-dependent reporter gene expression in THP-1 cells; this increase is inhibited by the p38-specific inhibitor FHP1, which is identical to SB 202190 (ref. 7). **d**, Expression of dominant-negative p38 (p38(M)) inhibits LPS-induced, MEF2C-dependent reporter gene expression in RAW cells. **e**, p38 is the main MEF2C kinase in LPS (10 ng ml⁻¹, 30 min)-stimulated RAW cells, as determined by an in-gel kinase assay. Depletion of p38 in the cell lysate was achieved by using anti-p38 polyclonal antibody.

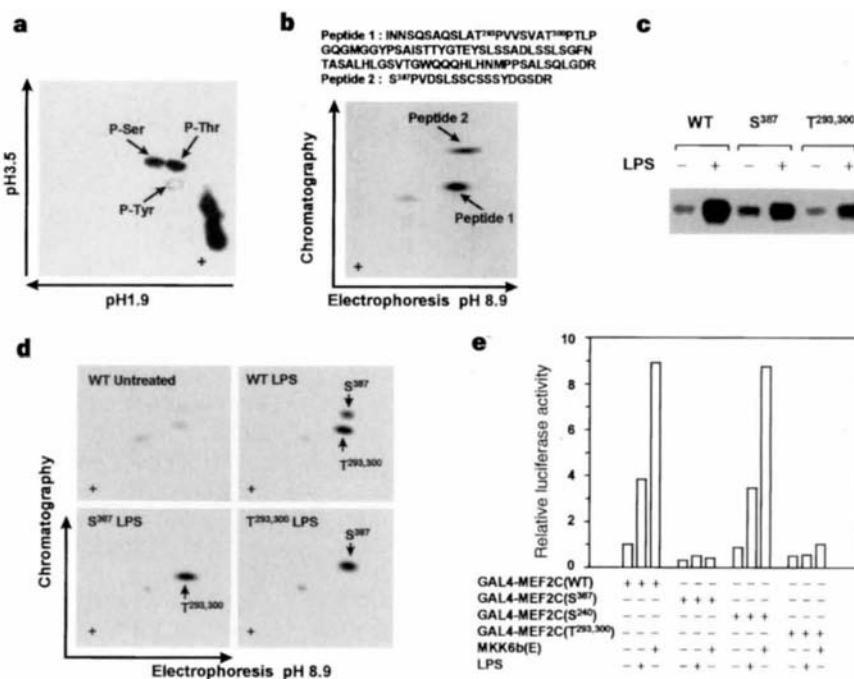
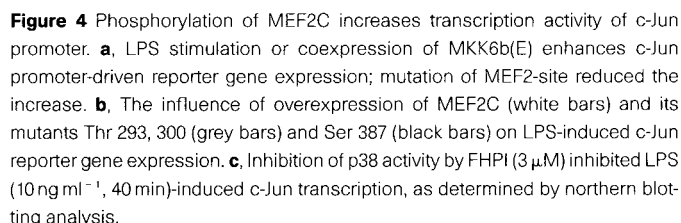


Figure 3 MEF2C transactivation activity increased by LPS is mediated by p38 through phosphorylation of MEF2C on Thr 293 (T²⁹³), Thr 300 (T³⁰⁰) and Ser 387 (S³⁸⁷). **a**, Phosphoamino-acid analysis of MEF2C phosphorylated by p38 *in vitro*. **b**, Phosphopeptide mapping of MEF2C phosphorylated by p38 *in vitro*. **c**, LPS induces phosphorylation of GAL4-MEF2C fusion protein and its mutants from

permanently transfected RAW 264.7 cells metabolically labelled with ³²P. **d**, *In vivo* phosphopeptide mapping of GAL4-MEF2C and its mutants. **e**, The influence of the mutation of phosphorylation sites of MEF2C on MEF2C-dependent reporter gene expression. Mutations at the phosphorylation sites of MEF2C reduced MKK6b(E)- or LPS-enhanced MEF2C-dependent reporter gene expression.

We know of no data that directly link MEF2C activation to expression of cytokine genes, such as that for tumour necrosis factor. However, p38-enhanced c-Jun expression might contribute to LPS-induced changes in AP-1 or the AP-1/CRE-like element binding activity in monocytic cells²⁰⁻²⁴, as the modulation of these DNA-binding activities by LPS requires *de novo* protein synthesis^{20,23}. Cytokine genes typically have AP-1/CRE-like elements. Thus, for infection with multiple exposures to microbial pathogens such as LPS, MEF2C activation might play an important role in controlling the balance of cytokine production through its effects on c-Jun expression. It has also been shown that stimulation with LPS leads to the activation of JNK and the subsequent phosphorylation of c-Jun²⁴. Thus LPS activation results in consumption of c-Jun protein, and p38-induced MEF2C activation results in increased transcription of the c-Jun gene, which itself would result in the replenishment of c-Jun protein. Because p38 and JNK are also quite often activated together by important phlogistic stimuli such as interleukin-1 or tumour necrosis factor, these two MAP kinase pathways may also be linked during many conditions in which inflammatory responses are upregulated. □



Methods

Yeast two-hybrid system. The two-hybrid screen, mating experiments, and X-gal colony filter assay were performed as described¹³.

In vitro binding of p38 and MEF2C. Glutathione-agarose beads bound with a GST fusion protein of wtp38 (GST-wtp38) were incubated with different amounts of MEF2C in buffer A (10 mM Tris-HCl, pH 7.9, 250 mM NaCl, 25 mM imidazole and 0.05% Triton-X-100) containing 2% BSA with or without cold ATP (20 μ M) for 3 h after blocking the nonspecific binding with 2% BSA in buffer A for 3 h. After the beads were washed 6 times with buffer A, they were subjected to SDS-PAGE followed by transfer to nitrocellulose; His-MEF2C was detected using Ni-NTA conjugated with alkaline phosphatase (Qiagen), and GST-p38 was detected with rabbit anti-p38 antibody together with peroxidase coupled to sheep anti-rabbit IgG (Cappel).

In vitro kinase assays. Equal amounts (2 μ g) of His-MEF2C, GST-c-Jun (residues 1–93)³ or MBP (Sigma) were used as substrates. His-tagged wtp38 (ref. 4), His-tagged ERK2 (ref. 25) or flag-tagged JNK1 (ref. 3) were used as kinases. The kinase reaction and quantification were performed as described^{3,26}. **EMSA.** Nuclear extracts of RAW 264.7 cells treated with or without LPS (10 ng ml⁻¹) for different times were incubated with a double-stranded, ³²P-labelled oligonucleotide containing a MEF2 binding site as a probe¹¹. An unlabelled MEF2 oligonucleotide probe and an oligonucleotide with a mutation in the MEF2 site (MEF2mut)¹¹ were used as competitors to determine the binding specificity.

Reporter gene assays. THP-1 cells were transfected with DEAE-Dextran²² and RAW 264.7 cells with calcium phosphate²⁷. Cells were transfected with a β -galactosidase expression vector pCMV- β , with the reporter plasmid pG5E1-bLuc, with an expression vector encoding a GAL4-MEF2C fusion protein or GAL4(1–147) or GAL4-MEF2C mutants, and with the expression vector encoding a constitutively active form of MKK6b(E) or with empty vector pcDNA3. In some experiments the cells were also transfected with increasing amounts of DNA (0, 2, 4, 8, 12 or 16 μ g) from an expression plasmid for p38(M). The total amount of DNA for each transfection was kept constant using pcDNA3. In studies using FHPI, the inhibitor was added (3 μ M) 36 h after transfection for 1 h; and the cells were then treated with or without LPS for 8 h. LPS (5 μ g ml⁻¹ and 10 ng ml⁻¹) was used to stimulate THP-1 and RAW 264.7 cells, respectively. The relative luciferase activities presented were normalized by dividing the luciferase activity by β -galactosidase activity.

Phosphoamino-acid analysis and phosphopeptide mapping. These methods were performed as described¹⁸. GAL4-MEF2C fusion protein and mutants from [³²P]orthophosphate-labelled permanently transfected RAW 264.7 cells (1 mCi ml⁻¹, 2 h) treated with or without LPS (10 ng ml⁻¹, 2 h) were immunoprecipitated with anti-GAL4 DNA-binding domain monoclonal antibody RK5C1 (Santa Cruz).

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Direct observation of the rotation of F₁-ATPase

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Cells employ a variety of linear motors, such as myosin^{1–3}, kinesin⁴ and RNA polymerase⁵, which move along and exert force on a filamentous structure. But only one rotary motor has been investigated in detail, the bacterial flagellum⁶ (a complex of about 100 protein molecules⁷). We now show that a single molecule of F₁-ATPase acts as a rotary motor, the smallest known, by direct observation of its motion. A central rotor of radius ~ 1 nm, formed by its γ -subunit, turns in a stator barrel of radius ~ 5 nm formed by three α - and three β -subunits⁸. F₁-ATPase, together with the membrane-embedded proton-conducting unit F₀, forms the H⁺-ATP synthase that reversibly couples transmembrane proton flow to ATP synthesis/hydrolysis in respiring and photosynthetic cells^{9,10}. It has been suggested that the γ -subunit of F₁-ATPase rotates within the $\alpha\beta$ -hexamer¹¹, a conjecture supported by structural⁸, biochemical^{12,13} and spectroscopic¹⁴ studies. We attached a fluorescent actin filament to the γ -subunit as a marker, which enabled us to observe this motion directly. In the presence of ATP, the filament rotated for more than 100 revolutions in an anticlockwise direction when viewed from the 'membrane' side. The rotary torque produced reached more than 40 pN nm⁻¹ under high load.

In the crystal structure of mitochondrial F₁-ATPase⁸, rigid¹⁵ coiled-coil α -helices of the γ -subunit penetrate the central cavity of the $\alpha_3\beta_3$ and extend into the stalk region that links F₁-ATPase to the F₀ portion. The amino terminus of the β -subunits is on the side opposite the stalk region of the γ -subunit. To fix the $\alpha_3\beta_3\gamma$ subcomplex on a glass plate, the subcomplex derived from a thermophilic bacterium was expressed in *Escherichia coli*, with ten histidines (His tag) linked to the N terminus of each β -subunit. The

glass plate was coated with horseradish peroxidase conjugated with Ni^{2+} -nitrilotriacetic acid (Ni-NTA), which has a high affinity for a His-tag and thus bound the subcomplex through the three β -subunits, with the F_0 side ('membrane' side) away from the glass (Fig. 1a). To visualize the rotation, γ -Ser107, which is presumably in the stalk region of the γ -subunit⁸, was replaced with cysteine, and α -Cys193, the only cysteine in the wild-type $\alpha_3\beta_3\gamma$ subcomplex, was replaced with serine by site-directed mutagenesis¹⁶; the introduced cysteine was biotinylated. A fluorescently labelled, biotinylated actin filament was attached to the γ -subunit through streptavidin, which has four binding sites for biotin.

Rotating actin filaments were found in the field of an epifluorescence microscope when 2 mM ATP was infused into a flow chamber containing actin-tagged $\alpha_3\beta_3\gamma$ subcomplexes on the bottom plate (Fig. 2). On average, one out of 70 filaments rotated continuously in one direction. Fifteen out of 70 showed only irregular to-and-fro fluctuation around one fixed point; these

were observed for at least 25 s, because fluctuating filaments in some cases started to rotate. Others were immobile, being attached to the glass surface at two or more points. Most of the rotating filaments had their rotation axis at one end of the filament (Fig. 2a), whereas some had the axis at the middle and rotated like a propeller (Fig. 2b). As the $\alpha_3\beta_3\gamma$ subcomplex was fixed to the glass plate through (presumably) three β -subunits and the actin filament was attached to the γ -subunit, this result shows that the γ -subunit rotates in the centre of the $\alpha_3\beta_3$ cylinder (Fig. 1a). The other subunits, δ and ϵ , are not necessary for the rotation; they may be a part of the stator and rotor, respectively¹⁷.

The time course of rotation of individual actin filaments is shown in Fig. 3. It is clear that the filaments rotate only in one direction. We observed 90 rotating filaments altogether and all, without exception, rotated anticlockwise when viewed from the membrane side (Fig. 1a). In the crystal structure⁸, the anticlockwise rotation of the central γ -subunit allows it to interact sequentially with the three

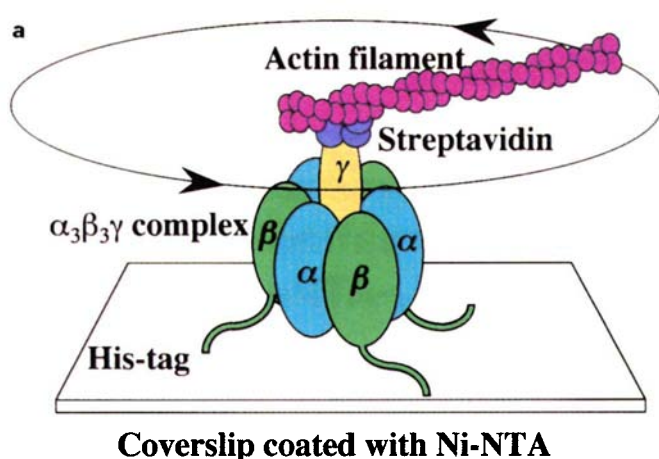


Figure 1 a, The system used for observation of the rotation of the γ -subunit in the $\alpha_3\beta_3\gamma$ subcomplex. **b**, Crystal structure of mitochondrial F_1 -ATPase⁸ viewed from the membrane side, or from above the glass plate in **a**. Only a part of the structure near the nucleotide-binding site is shown. The observed direction of the rotation of the γ -subunit is indicated by an arrow.

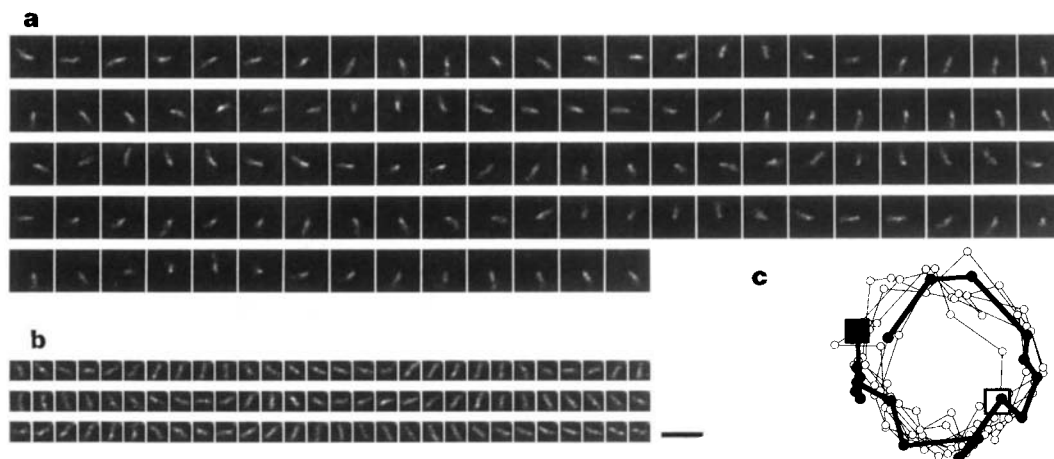
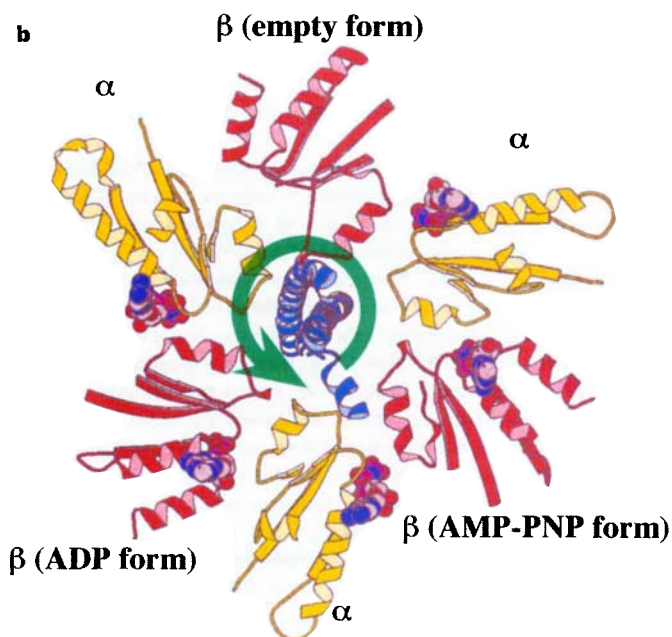


Figure 2 Sequential images of a rotating actin filament attached to the γ -subunit in the $\alpha_3\beta_3\gamma$ subcomplex. In the inverted microscope, the specimen is viewed from the bottom and its mirror image is formed on the camera²³. Therefore, images shown here correspond to the view from the top (Fig. 1a). **a**, A rotating filament with the rotation axis at one edge. Length from the axis to tip, 2.6 μm ; rotary rate, 0.5 r.p.s.; time interval between images, 133 ms. **b**, A rotating filament with the

rotation axis at the middle of the filament. Total length of the filament, 2.4 μm ; rotary rate, 1.3 r.p.s.; time interval between images, 33 ms. Scale bar, 5 μm in **a** and **b**. **c**, A trace of the centroid of the filament image in **a**. The trace starts at the filled square and ends at the open square. The first revolution is shown in the thick line.

forms of the β -subunits in the order: empty form, ADP-bound form, AMP-PNP (an ATP analogue)-bound form (Fig. 1b). This sequence is equivalent to the catalytic transition in one β -subunit in the order: ATP $\rightarrow$ ADP $\rightarrow$ empty forms, the order expected in the ATP hydrolysis reaction.

Figure 2 shows that the rotary speed was not constant and that even small, momentary reversals occurred occasionally. The fluctuation should in part be of brownian origin, but a certain angle-dependence was also noticeable (in Fig. 2a, for example, the filament tends to dwell in the bottom half, as in Fig. 2c). In filaments with lower average speeds, frequent pauses, accompanied by fluctuation, were observed (Fig. 3), often at the same angle(s). Obstruction by nearby proteins is a likely explanation, but intrinsic properties of this rotary motor may also contribute to these angle-dependent irregularities. In some cases, the angular distribution appeared to have three peaks separated by 120° , but precise analysis was hampered by the brownian fluctuation. Observation was terminated after a pause of >30 s or when the filament became completely immobile (39 out of the 90 cases), or when the filament was torn off near the attachment point and floated into solution (7/90), or after rotation continued for >1 min (44/90). Some filaments continued to rotate for >10 min.

When ATP was absent, there was no rotary motion apart from the brownian fluctuation (—ATP in Fig. 3), which, on rare occasions, accumulated over several minutes into a few turns in either direction. Azide, an inhibitor of the ATPase activity of F_1 -ATPase and of the $\alpha_3\beta_3\gamma$ subcomplex¹⁸, blocked rotation in the presence of ATP (+ATP + NaN_3 in Fig. 3). Because rotation was rare, we did an 'unpredicted test' in which we observed 1,800 filaments under each of the '+ATP', '—ATP' or '+ATP + NaN_3 ' conditions without knowing the identity of the sample. We made a complete search on 15 chambers, taking ~ 30 min for each chamber and stopping for 25 s at every actin filament that showed some sign of movement. We found that 25 filaments in '+ATP' made >5 turns during the first 25 s of observation. No filaments made >2 turns in 25 s under the '—ATP' or '+ATP + NaN_3 ' conditions.

The stoichiometry of three ATPase catalytic sites (on β -subunits) per single γ -subunit in the F_1 -ATPase indicates a rotary rate (without load) of 17 revolutions per second (r.p.s.), if the fixed sub-

complex hydrolysed ATP at the same rate as was measured in solution (52 ATP per second). The observed rate of filament rotation, on the other hand, was at most ~ 4 r.p.s. and lower for longer filaments (Fig. 3), suggesting that the γ -subunit carrying an actin filament rotated against a heavy load produced by hydrodynamic friction on the filament. Indeed, the torque needed to rotate an actin filament at the observed speed is quite large, >45 pN nm for the filament in Fig. 2a and >23 pN nm for Fig. 2b (see Methods). If this torque is produced at the β - γ interface at the radius of ~ 1 nm from the central axis of the $\alpha_3\beta_3$ cylinder⁸, the force that makes the γ -subunit slide past the β -subunit would amount to >45 pN (Fig. 2a) or >23 pN (Fig. 2b). By comparison, individual linear motors produce a sliding force of 3–6 pN (myosin^{1–3}), 5 pN (kinesin⁴), or 14 pN (RNA polymerase⁵). The slower movement under higher load is a feature common to all known linear motors and the flagellar motor. Whether the coupling between ATP hydrolysis and the rotation in F_1 -ATPase is loose¹⁹ as in linear motors, or as tight as in the flagellar motor²⁰, remains to be investigated.

Taken together, this work provides discriminating evidence for the physical unidirectional rotation of the γ -subunit in the F_1 -ATPase. It is now clear that the γ -subunit constitutes the rotating 'shaft' that mediates the energy exchange between the proton flow at F_0 and ATP synthesis/hydrolysis at F_1 -ATPase. Taking advantage of the single molecule observation system, we plan to analyse the torque and speed, mechanism of force generation, effect of ATP concentration, efficiency and architecture of the rotor and stator of this new type of enzyme, the smallest biological rotary motor known. □

Methods

Materials. The mutant (α -C193S, γ -S107C) $\alpha_3\beta_3\gamma$ subcomplex of thermophilic *Bacillus* PS3 was purified as described²¹. The subcomplex was treated with a 2–10 molar excess of 6-{N'-[2-(N-maleimido)ethyl]-N-piperazinyl-amido}hexyl-D-biotinamide (biotin-PEAC₃-maleimide, Dojindo) in 20 mM 3-[N-morpholino]propanesulphonic acid-KOH (MOPS-KOH, pH 7.0) and 100 mM KCl for 6 h on ice. Specific biotinylation of the γ -subunit was confirmed by western blotting with horseradish peroxidase avidin D (Vector Labs). Catalytic activities were measured at 25 °C at pH 7.0 in the presence of 50 mM KCl; the mutant subcomplex hydrolysed 52 ATP molecules per second,

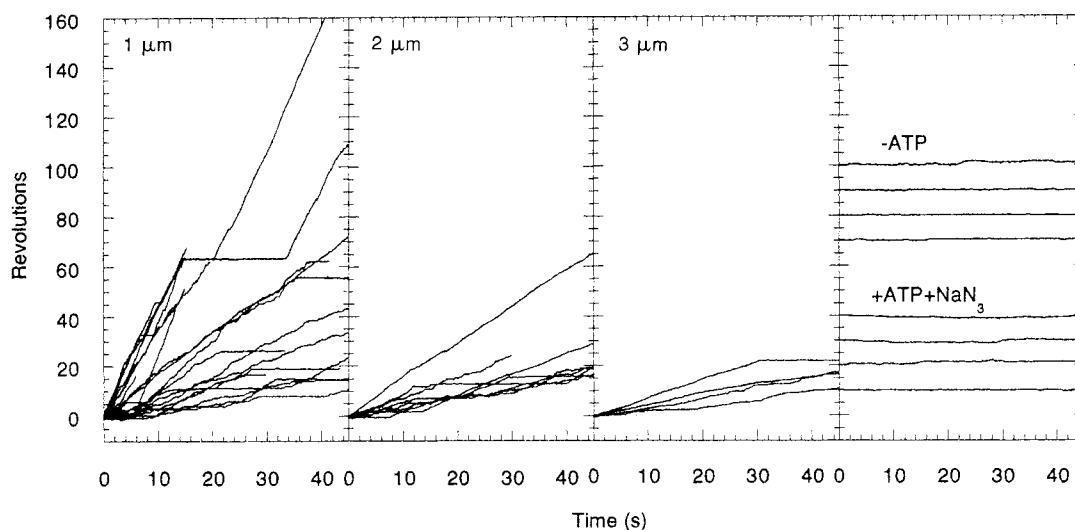


Figure 3 Time course of the rotation of the γ -subunit; each line represents one filament. The ordinate represents the number of anticlockwise revolutions. Rotating filaments, all in the presence of 2 mM ATP, are classified into three groups according to their length ('1 μm ' indicates 0.5–1.4 μm ; '2 μm ', 1.5–2.4 μm ; '3 μm ', 2.5–3.4 μm). Only those filaments that rotated around one end are shown, for which the rotating angles were conveniently estimated by centroid analysis as

in Fig. 2c. The angular resolution in these plots is estimated to be $\sim 20^\circ$, or worse in some cases, because the filament was not always straight and part of the filament occasionally went out of focus. '—ATP', without ATP; '+ATP + NaN_3 ', in the presence of 2 mM ATP and 10 mM NaN_3 ; for these control experiments, those filaments that moved most are selected and shown.

the wild-type subcomplex 52 ATP s^{-1} , and the native $F_1\text{-ATPase}$ 39 ATP s^{-1} . Rabbit skeletal actin ($30 \mu\text{M}$) was incubated with $150 \mu\text{M}$ biotin-PEAC₅-maleimide in 100 mM KCl , 1 mM MgCl_2 , 10 mM MOPS-KOH (pH 7.0) and $0.3 \text{ mM Na}_2\text{S}_2\text{O}_8$ at room temperature for 2 h. The actin was depolymerized in 2 mM MOPS-KOH (pH 7.0), 0.2 mM CaCl_2 and 2 mM ATP . Residual biotin was removed on a Sephadex G-25 column. Biotinylated actin ($5 \mu\text{M}$) was polymerized in $10 \text{ mM 2-(cyclohexylamino)ethanesulphonic acid-KOH}$ (pH 8.8), 100 mM KCl , 1 mM MgCl_2 and $5 \mu\text{M}$ phalloidin-tetramethylrhodamine B isothiocyanate conjugate (Fluka) overnight at 4°C , and crosslinked with $500 \mu\text{M}$ disuccinimidyl suberate (Pierce) at room temperature for 2 h. The reaction was quenched with 50 mM Tris-HCl (pH 8.8).

Immobilization of proteins. A flow cell for microscopic observation was constructed from a bottom coverslip ($24 \times 36 \text{ mm}^2$; Matsunami) coated with nitrocellulose and a top coverslip ($18 \times 18 \text{ mm}^2$), separated by two greased strips of Parafilm cover sheet. $0.6\text{--}1.2 \mu\text{M}$ of horseradish peroxidase-conjugated Ni-NTA (Qiagen) was introduced into the flow cell and allowed to adhere to the glass surface for 2 min. The cell was washed with buffer A (10 mg ml^{-1} BSA, 10 mM MOPS-KOH (pH 7.0), 50 mM KCl , 4 mM MgCl_2). Infusion and washing were repeated as follows: infusion of $10\text{--}100 \text{ nM}$ biotinylated $\alpha_3\beta_3\gamma$ subcomplex in buffer A (5 min), washing with buffer A, infusion of 180 nM streptavidin (Sigma) in buffer A (2 min), washing with buffer A, and infusion of 100 nM biotinylated fluorescent actin filaments in buffer A (5–15 min). The last wash was carried out with 0.5% 2-mercaptoethanol and an oxygen-scavenger system²² in buffer A containing, where indicated, 2 mM ATP or $10 \text{ mM Na}_2\text{S}_2\text{O}_8$. Observation started within 1 min of the beginning of the last washing. The actin filaments did not bind to the glass plate without the biotinylated subcomplexes. Also, the binding was dependent on streptavidin. In a control experiment, His-tagged subcomplexes fluorescently labelled at $\gamma\text{-Cys107}$ were fixed on a Ni-NTA surface and extensively washed with buffer A; subsequent washings with buffer A containing 50 mM imidazole (pH 7.4) removed $>85\%$ of the fluorescence. These results ensure that the actin filaments were attached to the biotinylated $\alpha_3\beta_3\gamma$ subcomplexes which were fixed to the glass surface through the histidine tags.

Observation of rotation. Actin filaments were observed under an epifluorescence microscope (Diaphot TMD, Nikon) with excitation and emission wavelengths at 546 nm and $560\text{--}620 \text{ nm}$, respectively. Images were taken with a CCD camera (Dage MTI) attached to an image intensifier (KS-1381, Videoscope), recorded on an 8-mm video tape, and analysed with a digital image processor (DIPS-C2000, Hamamatsu Photonics)^{2,23}. The frictional torque for the propeller rotation is given²⁴, in the simplest approximation, by $(\pi/3)\omega\eta L^3/[\ln(L/2r) - 0.447]$, where ω is the angular velocity, η ($10^{-3} \text{ N s m}^{-2}$) the viscosity of the medium, L the length of actin filament, and r (5 nm) the radius of the filament. For the rotation around one end of the filament, the torque is four times the above value. These values are actually underestimated, because the viscous drag near the glass surface is higher (up to $\sim 3\text{-fold}$ ²⁴ if all of the filament lies at a height of $(5 + 8) \text{ nm}$ from the glass surface, 5 nm being the filament radius and 8 nm the height⁸ of $\alpha_3\beta_3$) and because possible contact with the surface would produce additional friction.

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erratum

Spatio-temporal frequency domains and their relation to cytochrome oxidase staining in cat visual cortex

Doron Shoham, Mark Hübener, Silke Schulze, Amiram Grinvald & Tobias Bonhoeffer

Nature **385**, 529–533 (1997)

In this Letter, an editing error in the *Nature* office led to a misleading first sentence in the last paragraph. The complete paragraph should read as follows:

Tracing studies have shown that blobs in cat visual cortex are specifically connected to other visual cortical areas^{29,30} and that they receive a strong input from Y-cells in the lateral geniculate nucleus¹¹. Furthermore, we have preliminary evidence that low spatial frequency domains, like blobs, receive a stronger input from geniculate Y-cells³¹, which would be consistent with the preference for low spatial and high temporal frequencies revealed here. The blob and interblob regions of cat visual cortex, like their counterparts in primate visual cortex, appear to be compartments of parallel pathways that are specialized for analysing different attributes of the visual scene. □

correction

Transmission dynamics and epidemiology of BSE in British cattle

R. M. Anderson, C. A. Donnelly, N. M. Ferguson, M. E. J. Woolhouse, C. J. Watt, H. J. Udy, S. MaWhinney, S. P. Dunstan, T. R. E. Southwood, J. W. Wilesmith, J. B. M. Ryan, L. J. Hoinville, J. E. Hillerton, A. R. Austin & G. A. H. Wells

Nature **382**, 779–788 (1996).

In Table 2 of this Letter, the reported number of cases saved for policy 9 was erroneously given for 1996 to 2001 rather than for 1997 to 2001. The number should be 584 rather than 797. Thus, the number of cases saved in policies 11–14 should be reduced by 213. Also, the culling policy description for policy 6 should begin 'As 5)' rather than 'As 15)'.

An error caused Figs 1d and e in this article to be transposed. The legends are correct.

On page 783, in the first column in the sixth line of text 'F and G are two operators' should have been 'G and F are two operators'.

Finally, the manuscript on maternal transmission of the BSE agent in cows by J. W. Wilesmith *et al.* described in the paper as ref. 6, under consideration by *Nature*, has been withdrawn. □

Shed new light on cell biology

Fluorescence systems that allow cells in living organisms to be studied are among the products featured in this roundup. There's also a compact centrifuge that fits into a drawer, and the latest reagents, kits and instruments.



Biofuge vito has 24-place polypropylene rotor.

Biofuge vito

From Heraeus

A centrifuge designed to fit inside a drawer

To cater to scientists working within a limited area, Heraeus has developed the Biofuge vito, an ultra-compact centrifuge for microlitre applications. Designed to fit in a drawer under the laboratory bench, the centrifuge is easy to operate once the drawer is open. The rotor can be fitted and loaded as usual. To commence centrifugation, the drawer is pushed back until it locks, closing the lid of the centrifuge and allowing the user to activate the clear control keys. As the centrifuge is situated inside a drawer it is exceptionally quiet, even at its maximum speed of 13,000 r.p.m. Acceleration to a maximum RCF of 16,000g is said to be achieved within a few seconds and a 'quick-run' key provides short centrifugation runs, if required. The 24-place polypropylene rotor is autoclavable and resistant to most chemicals. The rotor holds 1.5- or 2-ml tubes and can be adapted to accommodate smaller tubes with a capacity of 0.2–0.6 ml. It is suitable for a diverse range of applications, including pelleting DNA and RNA, antibody and protein precipitation, and fractionation of bacterial and yeast cells.

Reader Enquiry No. 100

Imaging

Stereo fluorescence system

From Leica

Green-fluorescent protein (GFP) helps to give direct insight into the distribution, anatomy and development of certain structures in living cells and tissues

The bright-green fluorescing albumen molecule recently discovered in a species of jellyfish is revolutionizing work in molecular biology laboratories because of its versatile application possibilities and the ease with which the new techniques can be applied.



Leica stereo microscopes using GFP.

For example, it is now possible to study the migration of certain cells in living organisms (nematodes, fruit flies and zebra fish) *in vivo*, and to carry out investigations in real time. Leica stereomicroscopes are stated to produce three-dimensional images with greater depth of field than before, and so when combined with the powerful new fluorescence module they allow unprepared fluorescing specimens to be inspected, manipulated, sorted and recorded. The stereomicroscope is said to provide larger fields of view than a classical microscope at the same magnification and with fluorescence that is stated to be brighter. The intense light produced by the mercury vapour lamp, together with a filter set developed specifically for GFP, enable even the finest structures, such as individual nerve cells, to be differentiated. By virtue of its high 12.5:1 zoom range and its total magnification of $\times 160$ (with $\times 1.6$ planapochromatic objective and $\times 10$ eyepieces), the Leica MZ12 should prove useful for research in the fields of molecular cell biology, biochemistry and molecular pharmacology. The high numerical aperture (0.2) of the $\times 16$ planapochromatic objective and the associated resolution of 600 line pairs per millimetre expand the amount of information revealed by the incident-light fluorescence technique.

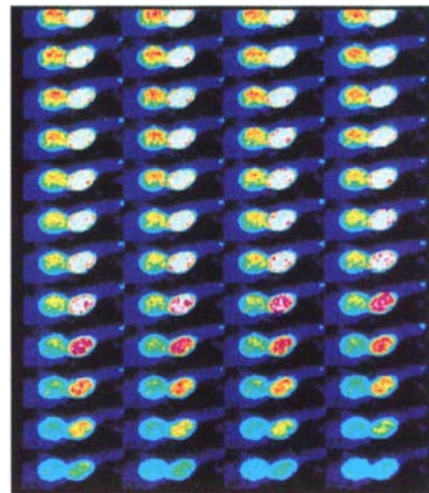
Reader Enquiry No. 101

Fluo-3 calcium indicator

From Bio-Rad

Stated to provides a 250-fold fluorescence enhancement between zero and saturating calcium concentrations

The kit allows calcium to be measured conveniently and accurately in confocal microscopy with a sensitivity that allows subtle intracellular calcium changes to be detected without using alternating wavelength techniques or ultraviolet optics. The high-resolution confocal imaging of calcium dynamics within optically thick cells and tissues is thus made possible using this kit. Fluo-3 AM reagent is stated to have a



Bio-Rad's fluo-3 calcium measurement kit.

purity of close to 100 per cent, enhancing the kit's accuracy in the range of 5–5,000 nanomolar free calcium. Applications include excitation-response coupling in cardiac myocytes and skeletal muscle, signal propagation in motor neurons and other nerve cells, as well as receptor-mediated signal transduction related to binding of drugs, toxins, antibodies and hormones. Fully compatible with common fluorescein filters, arc lamps and laser sources, the Fluo3 calcium indicator kit can be used with Bio-Rad's MRC 600, 1000 and 1024 confocal microscopes. The kit comprises a complete set of high-purity reagents and standardized buffers. These include high-grade fluo-3 AM reagent $\geq 95\%$ for loading whole populations of viable cells, fluo-3 pentapotassium salt for cell-free calibrations, a non-fluorescent calcium ionophore for extracellular and intracellular calcium equilibration, standardized buffers and a fluo-3 AM dispersing reagent.

Reader Enquiry No. 103

Openlab software

From Improvision

New imaging software for viewing cellular structure and function

Openlab has a modular design to provide a cost-effective means to meet the many needs of the biological research scientist, providing tools for scientific imaging that include image creation, presentation and publication. In addition, Openlab can be tailored to individual requirements with an extensive series of optimized modules, including digital confocal imaging at a fraction of the cost and without the difficulty



Open Lab from Improvision for cellular imaging.

associated with laser technology. The system comes with a versatile user interface, intracellular ion imaging for ratio-fluorescence imaging and quantitation of calcium and other intracellular ions. There is also fluorescence imaging, including merging multiple wavelength images and low-light level image optimization. Another feature is Automator, a tool designed for the simple and rapid production of custom experiment pathways.

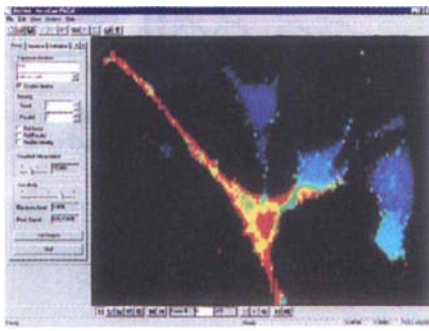
Reader Enquiry No. 104

NeuroCam

From AstroCam

A new camera for neurology studies

NeuroCam has been designed especially for the study of rapid neurological processes, such the propagation of electrical impulses along nerve cells. The design of the NeuroCam was inspired and refined in studies of the electric fields in single neurons. The camera consists of a 64×64 pixel CCD camera with thermoelectric cooling and high-precision electronics to extract very low noise 12-, 14- or even 16-bit images at 600 to 800 frames per second. A PCI bus into the standard PC memory is used to obviate the need for expensive frame grabbers. The manufacturer states that this camera range has a high dynamic range, giving up to 64,000 grey levels per pixel, allowing very small changes in signal



NeuroCam for studying neurological processes.

to be measured against high backgrounds, maximizing sample viability by reducing the need for high illumination levels.

Reader Enquiry No. 105

Concord

From Life Science Resources

An integrated system for imaging, photometry and electrophysiology data acquisition

This is a fully integrated microscope-based system, combining platforms for high-speed detection of ion-sensitive optical probes using high-resolution ratiometric imaging and sensitive photon counting. It provides complete electrophysiology data acquisition and experiment control with a workstation that allows sequential acquisition and analysis of spatial and temporal data from low-light-emitting optical probes reporting biological activity of single living cells or tissues. Applications include quantitative ion imaging and second-messenger detection, among others. Concord also facilitates integrated, time-locked electrophysiological studies from the same cells, which may be recorded simultaneously using patch clamp techniques. For these studies, the system can also be used to generate complex voltage or current command protocols for complete experimental control. Comprehensive optical engineering for imaging and photometry allows modular integration around a single epifluorescence microscope. A range of system options are available for multiple- or single-probe studies, with a choice of analog or digital CCD cameras (8- to 14-bit cooled with resolution from 192×165 pixels to $1,024 \times 1,024$ pixels). Dual- or single-wavelength photon counting at up to 2,000 samples per



Concord imaging from Life Science Resources.

second is provided as standard, as is the ability to interface and control any patch clamp amplifier. Excitation light can be provided either from the Rainbow filter wheel or the new SpectraMaster high-speed monochromator system. The workstation operates in a 32-bit Windows95 environment with analysis features that include montage, three-dimensional and pixel-line profiling.

Reader Enquiry No. 106

Reagents and kits

Predicta TNF- α kit

From Genzyme Diagnostics

Available in bulk, this kit comes in 3-, 4-, 5- and 10-plate bulk packs

The new ELISA has an improved sensitivity of 3 pg ml^{-1} , achievable in under 2.5 h, with an optional enhanced protocol that increases the sensitivity to 500 fg ml^{-1} . The assay procedure is said to be simple, with samples, standards and controls treated the same, thus removing the need for a final dilution factor. Human TNF- α can be measured in serum, plasma (EDTA, heparinized and citrated) and tissue culture fluids ($50 \mu\text{l}$ per well required). This provides a lyophilized standard, calibrated to NIBSC/WHO 87/650, breakaway wells and two levels of controls. No receipt interference is said to be detectable at ten times normal levels, however, the Predicta TNF- α ELISA kit has been shown to cross-react with porcine TNF- α .

Reader Enquiry No. 107

Potassium channel antibodies

From TCS Biologicals

A collection of potassium channel antibodies from Upstate Biotechnology and Alomone Laboratories

The Shaker subfamily of voltage-gated, delayed-rectifier K^+ channels is located mostly in the brain. Polyclonal antibodies against Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv1.5 are now available as are polyclonal antibodies to the GIRK family of K^+ channels (anti-GIRK1 and anti-GIRK2). Potassium ion channels are ubiquitous membrane proteins that occur in both excitable and non-excitable cells. Four major functional classes of K^+ channels can be distinguished: voltage-dependent K^+ channels, which are activated by membrane depolarization; Ca^{2+} -activated K^+ channels, which are primarily gated by intracellular calcium; inward rectifying K^+ channels; and Na^+ -activated K^+ channels. The molecular structure of K^+ channel α -subunits reveals the existence of four very different superfamilies: the Shaker archetype, the inward rectifier superfamily, the two-pore archetype of K^+ channels and the Isk or Mink archetype.

Reader Enquiry No. 108

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PROTEIN SEQUENCING

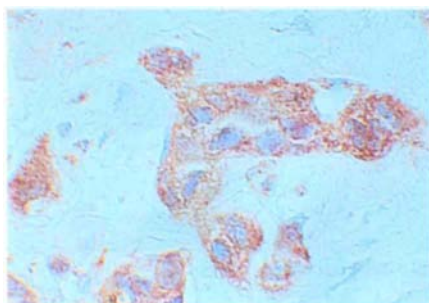
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READER ENQUIRY NO. 69



Antibody for apoptosis research from BioGenex.

Polyclonal antibody to bax protein

From BioGenex

Stains paraffin-embedded tissues expressing bax protein, which has been identified as a promoter of apoptosis.

The biochemical, physiological and morphological properties of apoptosis control are currently under intense investigation as the override of apoptotic control is suspected to cause or to contribute to some forms of carcinogenesis. Either under-expression of *bax* or overexpression of apoptosis inhibitors is observed in a number of disease states. In prostate carcinoma that has become refractive to androgen therapy, the normal expression of apoptosis inhibitor proteins is dramatically increased, while levels of *bax* protein remain high regardless of tumour grade. Studies of normal tissues demonstrate that the distribution of apoptotic regulator proteins correlates with the regenerative properties of the tissue studied. Epithelial cells of colon crypts show increases in *bax* expression as cells progress from the base of crypts to the luminal surface. The epitope-specific polyclonal antibody to *bax* protein can be used to stain *bax* in formalin-fixed, paraffin-embedded tissue sections. The antibody reacts specifically with an epitope (designated GPT) unique to the *bax* gene product. The photograph shows a paraffin section of breast carcinoma stained with anti-*bax* protein polyclonal antibody (GPT).

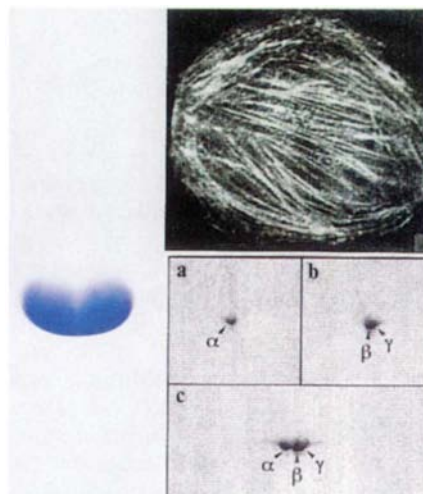
Reader Enquiry No. 109

Non-Muscle Actin

From Cytoskeleton

An injectable form of non-muscle actin labelled with rhodamine and the picture producing a fluorescence image

According to the manufacturer, non-muscle actin has not been commercially available until now. This actin is distinct from muscle actin, which many biochemical producers supply, by its association with many non-muscle cellular proteins. It is size chromatography purified to >97 per cent purity and it retains >95 per cent biological activity. This product is used in biochemical and cell biological studies that are based on



Cytoskeleton's non-muscle actin.

non-muscle cells like fibroblasts and neuronal cell types. The figure shows 100 µg of non-muscle actin (left), a mammalian cell microinjected with rhodamine non-muscle actin (top right) and two-dimensional gel isotype analysis. Cytoskeleton also offers a large range of cytoskeletal proteins conjugates and kits.

Reader Enquiry No. 110

ApoAlert Annexin V apoptosis kit

From Clontech Laboratories

Fast, early detection of apoptosis

The ApoAlert Annexin V apoptosis kit is based on observations that phosphatidylserine (PS) translocates from the inner to the outer surface of the cell membrane within two hours of the onset of apoptosis and that the annexin V protein has a strong affinity for PS. With ApoAlert, apoptotic cells are labelled by a simple incubation with an annexin V-FITC conjugate. This non-enzymatic assay is performed on living cells and takes less than an hour, allow apoptotic cells to be visualized by fluorescence microscopy or sorted by FACS.

Reader Enquiry No. 111

D-erythro-MAPP

From Kamiya Biomedical

A new inhibitor of alkaline ceramidase

d-erythroMAPP [(1S,2R)-d-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol], is now commercially available as an inhibitor of alkaline ceramidase. Ceramidases degrade ceramide, an important bioeffector lipid molecule involved in processes such as apoptosis, differentiation and growth regulation, into sphingosine and free fatty acids. d-erythro-MAPP inhibits alkaline ceramidase both in *vitro* and in cells, and it is a stated 100 times more potent (IC₅₀ of 1–5 µM) than available acid ceramidase inhibitors. Treated cells are stated to have significantly higher levels of ceramide with-

out concomitant increases in sphingosine. d-erythro-MAPP should prove to be a useful research tool that allows the effects of ceramide to be differentiated from those of sphingosine in a variety of cellular processes.

Reader Enquiry No. 112

Tfx-10, -20 and -50

From Promega

A new transfection reagent for eukaryotic cells

Tfx-10 and -20 have been teamed with Tfx-50, offering a trio of transfection reagents to help users select the reagent that performs best with a particular cell line. The trio is said to provide high-efficiency transfection of several types of eukaryotic cells, including HeLa, 293, K562, COS-7, CV-1, HIH/3T3, BHK, CHO and PC12 cells, and will transfect both in the presence or absence of serum in many cell lines. The single-tube protocol and short transfection times make the reagents easy to use and optimize, according to the manufacturer. Applications include stable or transient DNA transfection of eukaryotic cells, and transfection of RNA and oligonucleotides.

Reader Enquiry No. 113

PLA2 substrate

From Cayman Chemicals

A new substrate that provides a convenient, sensitive and continuous spectrophotometric assay of PLA2 activity

Phospholipase A2 (PLA2) is an important enzyme in many signal transduction pathways, catalysing the hydrolysis of fatty acids from the sn-2 position of phospholipids. The most common assay used at present involves radiolabelled *Escherichia coli* membranes as a substrate. However, in addition to requiring the use of radioactivity, this assay is labour intensive and costly. A new substrate, dithioheptanoyl phosphatidylcholine, provides a convenient, sensitive

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and continuous spectrophotometric assay of PLA2 activity. Upon cleavage of the thioester bond at the sn-2 position by PLA2, free thiols are detected using DTNB (Ellman's reagent). The substrate works with all forms of PLA2 with the exception of cytosolic PLA2.

Reader Enquiry No. 114

Instrumentation

IASys Auto + Biosensor

From Affiniti Sensors

Increased throughput using kinetic analysis as an alternative to equilibrium or end-point studies using conventional methods such as ELISAs, and other biosensors

Newly introduced, easy-to-use software and real-time data analysis further extends the uses of this system. IASys Auto+ can be used to characterize binding in these complex interactive systems. Results obtained from the system are more informative, and experiments are said to be quicker to perform and are simplified by the absence of labels. It analyses and displays data instantly and its Adaptive Sequence Kontrol (ASK) software allows for results-based decisions in response to simple 'if/then' commands. ASK enables immediate control of experimental parameters, such as sample contact times, reagent and sample sequence, or sample recovery, resulting in increased sample throughput and faster optimization of data collection for more accurate kinetic analysis. For example, when using IASys Auto+ to study monoclonal anti-HSA binding to HSA immobilized on CM-Dextran, the sample throughput is increased by including time and response cut-off values for the binding signal.

Reader Enquiry No. 115



IASys Auto+ biosensor system.

TD-20/20 luminometer

From StepTech

A research luminometer, offering improved sensitivity and versatility

It can measure light output in the unique sample compartment, which can accommodate an 8×50-mm test tube, 1.5-ml microfuge tube, 28-mm scintillation vial, a 35-mm culture dish and solids of various shapes and sizes. Also accommodating wavelength-specific filters and range extender, the luminometer is sensitive to 0.1 fg of luciferase with a wide dynamic range of 10³ or greater, easily extended to 10⁷ or greater with a range extending optical filter. An optional accessory is the internal auto injector which is designed for routine laboratory work, allowing the system to inject variable amounts of reagents (from 50–250 µl) with reproducible precision and accuracy. Injection timing is adjusted with the TD-20/20's programmable injection delay. Output, in ASCII, can be transmitted via the instrument's RS232 port to any PC, and it will also interface with Epson-compatible printers. Lastly, the footprint is small, measuring just 23.5 cm wide × 28 cm deep × 21 cm high.

Reader Enquiry No. 116

FluoroCount

From Packard Instruments

A microplate fluorometer instrument for reading of fluorescent assays in 6-, 12-, 24-, 48- or 96-well microwell plates

FluoroCount can be used to read a wide variety of assays, including immunoassays, reporter gene assays, oxidative burst/phagocytosis, ion-channel monitoring, cytoproliferation/cytotoxicity, cell-adhesion and protease assays, as well as to perform protein or nucleic acid quantification. It has flexible configurations to optimize performance for specific assays. According to the manufacturer, it is the only microplate fluorometer available with an external, fibre-optic light source, which allows the user to substitute other light sources, such as xenon or lasers, according to assay requirements. Plates can be read from above or beneath the well. The instrument uses digital photon integration to fine-tune its sensitivity. The FluoroCount is supplied with Plate-Reader for Windows and I-Smart data analysis software.

Reader Enquiry No. 117

FL600 fluorescence and absorbance

From Bio-Tek Instruments

A microplate fluorescence and absorbance reader that has a dual-probe optical system

This system enables top and bottom epifluorescence reading of microplates with software that optimizes top and bottom reading, improving the sensitivity of immunoassays



Bio-Tek's microplate reader.

and cellular assays with a variety of plasticware that includes 6- to 384-well configurations and Terasaki plates. The dual-probe optical system also enables the reading of assays measured by absorbance, such as ELISAs, MTT, protein and other colorimetric assays, providing two detection technologies in one machine. The FL600 is controlled by the company's KC4 for Windows data reduction software package.

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These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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